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Activation of Latent Mouse Pneumonitis Virus by Human Serum.*

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During an investigation of specimens from a patient with primary atypical pneumonia, pleural fluid was inoculated intranasally in albino Swiss mice. Many of these animals showed extensive pulmonary consolidation, during the second week after inoculation. When impression films of the consolidated areas were stained by the Macchiavello method, numerous red-staining intracellular granules were seen which bore a close morphological resemblance to the elementary bodies of psittacosis.

Serum freshly obtained from this patient was also inoculated intranasally in mice, and the majority developed a similar type of pneumonitis. Subsequently, several samples of serum freshly obtained from normal persons were inoculated in mice. Almost all of the mice which received normal human serum intranasally were found to have developed pulmonary lesions which were indistinguishable from those produced by the pleural fluid. Moreover, structures resembling elementary bodies were seen in impression films prepared from many of their lungs. Similar lesions

* The Bureau of Medicine and Surgery of the United States Navy does not necessarily undertake to endorse views or opinions which are expressed in this paper.

were not encountered in the lungs of normal uninoculated mice. When either the pleural fluid or the serum was heated at 56°C for 30 minutes and then inoculated, no pulmonary lesions were produced in mice.

Because of the possible relation of these observations to the general problem of latent viruses, and also because of their obvious importance in relation to the interpretation of results of attempts to recover viruses from human material by the intranasal inoculation of mice, this phenomenon was studied further.

Samples of fresh human serum from 6 normal adults were each inoculated intranasally into groups of mice under light ether anesthesia. After 8 days some of the mice were killed, and in about 80% of the animals the lungs were found to contain irregular circumscribed areas of greyish or pearly, and often lobulated, consolidation, varying in extent from pinhead-sized foci to lesions involving the major portions of 4 lobes. The lesions were found usually at the hilar region, and extended outwards therefrom, but sometimes occurred also at the periphery. Similar areas of consolidation were seen in the lungs of the remaining mice which were killed after 16 days, although in most instances the pneumonitis was less extensive at this time. Impression films prepared from the cut surface of the pulmonary lesions, stained by the Macchiavello technic, showed structures resembling elementary bodies in about one-half of the preparations studied. These bodies were sometimes present in abundance, but more often a prolonged search was necessary before they could be found. The microscopic pathology of the pulmonary lesions was characterized by areas of dense parenchymal infiltration with polymorphonuclear cells, and lesser numbers of cells of the mononuclear series. The smaller bronchi and alveoli were often filled with cellular exudate which consisted chiefly of polymorphonuclear cells.

Transmission of the disease in mice was attempted by the inoculation of 10% suspensions of consolidated lung tissue intranasally, intracerebrally or intraperitoneally in other groups of mice. No evidence of disease resulted from either intracerebral or intraperitoneal inoculations. In many instances, intranasal inoculation also gave negative results, or produced areas of consolidation in only a small percentage of animals. On 3 occasions, however, intranasal passage resulted in pulmonary lesions in about 50% of inoculated mice, and in 2 instances it was possible to continue transmission of the disease for 3 serial passages. In both series the 4th passage yielded negative results. Attempts to transmit the disease directly from mice to cotton rats, guinea pigs and Syrian hamsters were unsuccessful.

Suspensions of consolidated lung tissue from mice which had received serum were inoculated on the chorioallantoic membrane of 8-day-old chick embryos, and serial passages were made from embryo to embryo by this route at 3-day intervals. A total of 12 different pools of consolidated lung tissue from mice which had received normal human serum were passed in this manner. From 2 of these pools (No. 6 and No. 7) an elementary body virus was obtained after the third membrane passage. This virus was morphologically indistinguishable from the virus of psittacosis.

When 10^{-1} or 10^{-2} dilutions of suspensions of infected embryo membranes were inoculated intranasally in mice, a fulminating pneumonitis resulted which caused death of all animals within 36 hours. Higher dilutions of the membrane suspensions, *i.e.*, 10^{-3} through 10^{-6}, produced only chronic infections in mice, and extensive pulmonary lesions were still present 6 weeks after inoculation. The pathology of the pulmonary lesions produced appeared to be identical with that seen after infection by either the virus of mouse pneumonitis (Nigg[1]), or cat pneumonitis (Baker[2]). Both of these latter agents have been shown to be members of the psittacosis-lymphogranuloma group.[3,4]

The embryo passage virus, like the Nigg and Baker viruses, was pathogenic for mice only when inoculated intranasally. Intracerebral or intraperitoneal inoculations with high con-

1 Nigg, C., *Science*, 1942, **95**, 49.

2 Baker, J. A., *Science*, 1942, **96**, 425.

3 Eaton, M. D., and Corey, M., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 165.

4 Thomas, L., and Kolb, E. M., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 172.

TABLE I.
Results of Cross Complement Fixation Tests with Strains No. 6 and No. 7, and Members of the Psittacosis-Lymphogranuloma Group of Viruses.

Immune serum against	Complement fixation titer against indicated antigen					
	Strain No. 6	Strain No. 7	Psittacosis virus	Baker virus	Nigg virus	Normal yolk sac
Strain No. 6	1:128	1:128	1:64	1:64	—	0
" " 7	1:16	1:16	1:16	1:8	1:8	0
Psittacosis virus	1:256	1:256	1:256	1:256	1:256	0
" "	1:64	1:64	1:64	1:16	1:32	0
Baker "	1:64	1:64	1:128	1:128	1:128	0
Nigg "	1:16	—	1:8	1:16	1:64	0
Normal serum	0	0	0	0	0	0

centrations of the virus produced no evidence of disease, and the virus could not be detected in either the brains or spleens of mice following such inoculations. The embryo passage virus was pathogenic for both cotton rats and hamsters when inoculated intranasally, but not by other routes.

Hamster lung tissue containing the virus was employed for the immunization of hamsters by both the intranasal and intraperitoneal routes. Following immunization antisera were obtained which in high dilution were capable of fixing complement with yolk sac antigens prepared from either of the 2 embryo passage strains.

Cross complement fixation tests with other members of the psittacosis-lymphogranuloma group of viruses were carried out. Yolk sac antigens as well as hamster antisera against the Nigg virus, the Baker virus, and the two embryo passage strains (strains No. 6 and No. 7) were prepared by methods which have been described previously.[4] Psittacosis virus antigen was prepared from chick embryo tissue culture by the method used as a routine in this laboratory.[5] Antiserum against psittacosis virus was obtained from immunized mice, as well as from human patients convalescent from psittacosis.

The results of these cross complement fixation tests are shown in Table I. Sera from animals immunized with either strain of virus, obtained from mice inoculated with human serum, fixed complement with the homologous as well as with each of the heterologous virus antigens tested. Moreover, antigens prepared from each of these strains caused fixation of complement in the presence of either the homologous or the heterologous antisera. These results indicate that both strains of virus are related antigenically to the Nigg, Baker, and psittacosis viruses.

The factor in certain human sera which leads, on intranasal inoculation, to the development of pneumonitis in mice has not been identified, nor is the mechanism by which this pneumonitis is induced understood. This serum factor was found to be thermolabile, and was destroyed completely by heating at 56°C. Attempts to restore the property to heated sera by the addition of unheated serum from either mice or guinea pigs were unsuccessful. That the factor may be present in the serum of species other than human beings is suggested by results obtained in preliminary experiments with ferret serum. Pneumonitis was produced in mice by intranasal inoculation of ferret serum in dilutions as high as 1:16.

Summary and Conclusions. The intranasal inoculation of fresh human serum in mice resulted in the development of pulmonary consolidation in a high percentage of animals. Structures resembling elementary bodies were seen in impression films prepared from these lesions. Serial passage in mice yielded irregular results, although in 2 series consolidation recurred in each of 3 passages. Serial passage on the chorioallantoic membrane of chick embryos resulted in the isolation of 2 strains of a pneumotropic elementary body virus. Cross complement fixation tests demonstrated that this virus was related antigenically to other members of the psittacosis-lymphogranuloma group of viruses. These observations suggest the possibility that this virus

[5] Smadel, J. E., *J. Clin. Invest.*, 1943, **22**, 57.

may be present in latent form in a considerable number of laboratory mice. The finding that the virus can be activated by the intranasal inoculation of fresh human serum seems of considerable interest.

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Effect of Sulfonamides on Toxic and Antigenic Actions of Endotoxins of Certain Gram-Negative Bacteria.

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Four independent groups of workers[1–4] have reported that sulfonamide compounds protect mice to a limited extent against the lethal effects of various bacterial toxins, especially the endotoxins* of gram-negative bacteria. On the other hand, some workers[5–7] failed to observe protective effects in similar experiments.

Hutner and Zahl[3] found that orally administered sulfanilamide protected mice against between one to ten LD_{50} of the endotoxin of *Salmonella typhimurium*. The object of the present study was: (1) to obtain a quantitative evaluation of the relative degree of protection provided by sulfanilamide, sulfathiazole, sulfapyridine, sulfadiazine, sulfamerazine, and sulfaguanidine,† (2) to ascertain if this protective action of the sulfonamides affects the immunizing properties of the endotoxins of gram-negative bacteria, as determined by the degree of resistance to the endotoxin developed by mice immunized while receiving sulfonamide treatment.

Relative Protection by Various Sulfonamides. The partially purified endotoxins of *Salmonella typhimurium* and *Shigella paradysenteriæ* Flexner were chosen as representative of endotoxins of gram-negative bacteria generally. These organisms were grown in large quantities in synthetic culture media, using methods described elsewhere.[8] The

1 Levaditi, C., and Vaisman, A., *C. R. Soc. Biol.*, 1938, **128**, 463; Levaditi, C., Vaisman, A., and Reinié, L., *Ann. Inst. Pasteur*, 1938, **61**, 635.

2 Carpenter, C. M., Hawley, P. L., and Barbour, G. M., *Science*, 1938, **88**, 530; Carpenter, C. M., and Barbour, G. H., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 255; Carpenter, C. M., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 354; Carpenter, C. M., *Rep. Proc. 3rd Internat. Congress Microbiology*, 1940, 595.

3 Hutner, S. H., and Zahl, Paul A., *Science*, 1942, **96**, 563; Zahl, Paul A., and Hutner, S. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 285.

4 Birger, O. G., and Hal'perin, E. P., *Zeit. Mikrobiol., Epidemiol., Immun.* (U.S.S.R.), 1941, **5-6**, 94.

* For most gram-negative bacteria, the toxic factor present in the bodies of the organisms is probably part of the toxic principle somatic O antigen. In *Neisseria*, some species of *Hemophilus* and *Brucella*, and possibly the rough phase of most gram-negative bacteria, the toxin may be part of a nucleoprotein, different in chemical pattern from the O antigens studied by Boivin, Morgan and Partridge, and others. However, the mode of pathogenesis of both toxins appears similar, if not identical. We have therefore for these studies preferred the more inclusive term "endotoxin" or "toxic factor" rather than the more precise immunochemical descriptions.

5 Long, P. H., and Bliss, E. A., *The Clinical and Experimental Use of Sulfanilamide, Sulfapyridine, and Allied Compounds*, 1939, Macmillan Company, New York City.

6 Buttle, G. A. H., Parish, H. J., McLeod, M., and Stephenson, D., *Lancet*, 1937, **1**, 681.

7 Gross, P., Cooper, F. B., and Lewis, M., *J. Inf. Dis.*, 1938, **63**, 245.

† We wish to express our appreciation to Lederle Laboratories, Inc., and to the American Cyanamide Company for generous gifts of sulfanilamide, sulfathiazole, sulfapyridine, and sulfadiazine, and to Sharp and Dohme for sulfamerazine.

8 Zahl, Paul A., and Hutner, S. H., *Am. J. Hyg.*, in press.

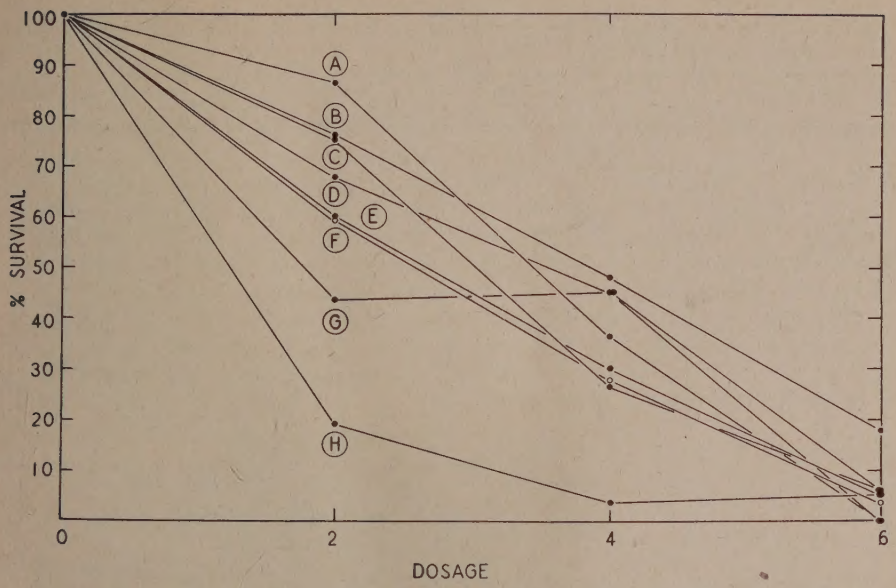

FIG. 1.

Effect of orally administered sulfonamides on the survival of mice receiving injections of *Salmonella* and *Shigella* endotoxins. Dosage is LD_{50} defined as amount of endotoxin required to kill within 24 hours 50% of mice injected intraperitoneally. In dry weight the amount of toxic material constituting 1 LD_{50} for *Salmonella* is 2.2 mg; for *Shigella*, 8.0 mg.

A—sulfanilamide; B—sulfaguanidine; C—sulfathiazole; D—sulfadiazine; E—sulfamerazine; F—sulfanilamide (*Shigella*); G—sulfapyridine; H—controls. The data of curve (F) is for mice receiving sulfanilamide following the injection of *Shigella* endotoxin. The remainder of the data applies to *Salmonella*.

Each plotted point represents 16 to 38 animals. The control points represent 50 to 76 animals. The total data plotted represent 659 mice.

cultures were killed by adding phenol to 2%, reduced in volume by pervaporation, precipitated from 66% acetone, and dried with acetone and ether. Aqueous dispersions of the precipitates, injected intraperitoneally, were used for all tests.

Control data on the survival of mice after the injection of the endotoxins at 3 dosage levels were obtained by observing the number of mice surviving at the end of 24 hours. The dosage levels chosen were 2, 4, and 6 LD_{50}. The LD_{50} (see caption to Fig. 1) is based on dose-survival curves published elsewhere.[8] The protective effects of the sulfonamides on the endotoxin-treated mice was determined by administering the sulfa drugs by means of a stomach tube attached to a tuberculin syringe, and observing the number of mice surviving at the end of 24 hours. In view of the relative absence of toxic effects from large doses of the sulfonamides in mice, an arbitrary dose of 25 mg of the sulfonamides in aqueous suspension was selected. The sulfonamides were administered a few minutes before the intraperitoneal injection of the endotoxin. The number of mice surviving for 24 hours provided the data plotted in Fig. 1.

Immunization During Sulfonamide Treatment. To determine whether the sulfonamides interfere with the immunizing properties of the *Salmonella* endotoxin while exerting protection against the toxic material, the following experiments were performed. In Experiment 1 the mice of Group A (50 male Rockland mice of 18-20 g) were given injections of the endotoxin in increasing doses according to the schedule listed in Table I. The number of animals surviving each injection was recorded. Several days after the last injection on the immunizing schedule, a very heavy dose (10 LD_{50}—lethal to all the controls) was injected. The survival data follow-

TABLE I.

Survival of Mice Receiving Sulfathiazole Treatment While Being Immunized with the Endotoxin of *Salmonella typhimurium*. Exp. No. 1, Group A, is control (without sulfathiazole) series; Exp. No. 1, Group B, is series receiving sulfathiazole during immunization. Exp. No. 2 is similar to No. 1, except that the dosages for immunization are higher and the schedule is somewhat altered. For definition of LD_{50} see caption to Fig. 1.

	Group A			Group B		
Days after 1st inj.	Dose of *Salmonella* endotoxin, LD_{50}	Dose of sulfathiazole (oral)	No. of mice surviving preceding inj.	Dose of *Salmonella* endotoxin, LD_{50}	Dose of sulfathiazole (oral) mg	No. of mice surviving preceding inj.
			Exp. No. 1.			
0	1/8	—	50	1/8	25	50
2	1/2	—	48	1/2	25	50
5	1	—	40	1	25	48
7	1	—	38	1	25	46
9	2	—	35	2	25	44
13	4	—	32	4	—	44
17	10	—	28	10	—	38
18	—	—	11	—	—	16
			Exp. No. 2.			
	Group C			Group D		
0	1	—	50	1	25	50
2	2	—	31	2	25	44
5	4	—	24	4	25	40
7	4	—	16	4	25	34
12	10	—	14	10	—	32
13	—	—	5	—	—	9

ing this massive injection was taken as an index of the degree of immunity which had developed in these control animals. Concurrently another series of 50 animals (Group B) was set up in which the mice, in addition to receiving the immunizing injections of endotoxin, were given 25 mg oral doses of sulfathiazole. This dose of sulfathiazole accompanied each immunizing injection, but was *not* given with the final massive assay injection.

In Experiment 2 the above steps were repeated but at higher dosage levels of immunizing injections. The data for the control (Group C) and for the sulfathiazole-treated animals (Group D) are also listed in Table I.

Results and Discussion. One may conclude from Fig. 1 that the 6 sulfonamides tested confer definite but limited protective effects of comparable magnitude against the lethal action of the *Salmonella* endotoxin, and that sulfanilamide at least has a similar effect on the *Shigella* endotoxin.

The failure of several investigators[5–7] to observe a protective effect of the sulfonamides on bacterial endotoxins is difficult to explain, except possibly on the basis that the effect, being small, might have been overlooked unless large numbers of animals were used and doses carefully controlled. It is apparent from our experience that if the assay dose had been in excess of 4-6 LD_{50}, the protective effect of the sulfonamides would probably have been missed. In addition much of the data cited by these workers concerns toxins from gram-positive organisms. The question of protection against toxins of gram-positive organisms falls outside the scope of this paper.

It appears probable that the protective action of the sulfonamides against the toxic preparation used in our experiments is largely referable to the O antigen component, and not to unidentified impurities. This conclusion is based: (1) on the demonstration by Boivin and Mesrobeanu[9] and other workers that the principal toxic component of the cells of gram-negative bacteria in the smooth phase is found in the O antigen, (2) on the reports of Levaditi, Vaisman, and Reinié[1] of a protective effect of sulfonamides against highly purified O antigens of gonococcus, meningococcus, *Salmonella*, *Shigella* and *Pasteurella*

[9] Boivin, A., and Mesrobeanu, L., *Ann. Inst. Pasteur*, 1938, **61**, 426.

prepared according to Boivin's trichloracetic acid technic.

The protective effect of sulfonamides appears to admit of at least 2 possible explanations: (1) that the action is directly on the toxic O antigen, *i.e.*, the sulfonamides participate in a detoxication process, (2) that the action is one of enhancing the general resistance of the body to the toxic material. The possibility of a direct effect of sulfonamide on the endotoxin appears to be ruled out by the reports of both the Levaditi and Carpenter groups that the toxicity of the endotoxin was not permanently reduced after incubation *in vitro* with sulfonamides.

While this conclusion appears to oppose the hypothesis that a detoxication process is involved in the effect of sulfonamides on the action of gram-negative endotoxins, the hypothesis cannot on this basis be dismissed entirely; for even if a major portion of the antigen were removed by sulfonamide detoxication, enough could conceivably have remained to elicit the observed antibody response. It must be borne in mind that we did not determine the minimal amounts of antigen required for immunization.

An examination of Table I indicates that a comparable degree of immunity was developed in mice subjected to a particular course of immunization, irrespective of whether sulfathiazole was administered concurrently (Groups B and D) or was not administered (Groups A and C). Mason[10] reported that rabbits undergoing immunization with pneumococci while being treated with sulfonamides developed the same antibody titer as rabbits not receiving sulfonamide treatment. Finland, Spring, and Lowell[11] obtained similar results in human pneumococcus pneumonia when sulfapyridine was used. We conclude from these observations that the sulfonamides did not influence the antigen-antibody reaction in respect to the gram-positive pneumococci. Our experiments indicate a similar lack of interference with immunization against the endotoxins of gram-negative organisms.

Our previous work[12,13] indicates that a similar mode of action characterizes the endotoxins of almost all gram-negative bacteria; and it is generally considered that the sulfa drugs, while varying in clinical efficacy, are also similar in mode of action. Although the experimental work reported here was confined to a test of *Salmonella* and *Shigella* endotoxins, and to a limited number of sulfa drugs, it is reasonable to expect that similar results would have been obtained with other sulfa drugs and with the endotoxins of other gram-negative bacteria.

In view of the protective action of sulfonamides against endotoxins of gram-negative organisms and the absence of gross interference with the immunization process, at least in so far as immunity to the endotoxin is concerned, there is a possibility that sulfa drugs may be of clinical value in mitigating certain undesirable symptoms which often accompany the use of typhoid, dysentery, and other vaccines prepared with gram-negative organisms.

Summary. Orally administered sulfanilamide, sulfathiazole, sulfapyridine, sulfadiazine, sulfamerazine, and sulfaguanidine confer an equivalent but limited protective effect against the lethal action of intraperitoneally-administered endotoxin of *Salmonella typhimurium*. Sulfanilamide has a similar effect on the endotoxin of *Shigella paradysenteriæ* Flexner. Sulfathiazole administered during the immunization of mice with *Salmonella* endotoxin appears not to interfere with the immunizing process, as determined by degree of resistance to the lethal action of the endotoxin. Reasons are advanced to support the view that these results apply to other sulfonamide drugs and to the endotoxins of gram-negative bacteria generally.

[10] Mason, M. M., *Vet. Med.*, 1942, **37**, 30.

[11] Finland, M., Spring, W. C., and Lowell, F. L., *J. Clin. Invest.*, 1940, **19**, 179.

[12] Zahl, Paul A., Hutner, S. H., Spitz, S., Sugiura, K., and Cooper, F. S., *Am. J. Hyg.*, 1942, **36**, 224.

[13] Hutner, S. H., and Zahl, Paul A., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 364.

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A Toxic Factor in Tissues in Cases of Nonspecific Ulcerative Colitis.*

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A substance which is toxic and often lethal for mice has been extracted from the liver and lungs of persons who had nonspecific ulcerative colitis.

Methods. The lungs and livers of persons who died of nonspecific ulcerative colitis, as well as numerous other control diseases, were saponified and the nonsaponifiable fraction[1] was tested for toxicity by subcutaneous injection into mice. The tissues were obtained at necropsy, chilled to ice box temperature immediately thereafter, and preserved by freezing or in a volume of ethanol equal in ml to the weight of the tissue in grams. The animals were of both sexes, of various ages, and of 3 different strains. These 3 factors seemed to have no influence on the results of the tests for toxicity. The extracts were suspended in a fatty solvent. The liver extracts were suspended in sesame oil so that the dose of 500 mg injected into each mouse was contained in a total volume of 1.0 cc. The lung extracts were administered in a dose of 250 mg per mouse, made up in tricaprylin so that the final volume was 1.0 cc. The number of mice used in each experiment was governed by the amount of extract available; it was, of necessity, small.

Results. The toxic factor was recognized by the condition which it induced in the mice, characterized by shock, collapse, and coma, with or without twitching and convulsions, usually terminating fatally. The first sign given by the mice was often scratching of the nose or twitching of some part of the body. Sometimes they lapsed gradually into coma. In other experiments the reaction was ushered in with excitement and intermittent convulsions. The symptoms began between five minutes and one hour after injection, and resulted fatally in from 10 minutes to 4 hours; death usually occurred between 30 and 60 minutes after injection. Mice which had been in deep coma sometimes recovered, regaining normal behavior between the third and fourth hours.

Necropsies revealed no recognizable anatomical abnormality. The lungs were not over-inflated, and on microscopical examination they showed no fat embolism.

Three out of 4 extracts obtained from livers in cases of nonspecific ulcerative colitis were highly toxic, producing shock in every mouse and death in nearly all. (See Table I.) Controls consisted of extracts prepared from 23 livers, representing a variety of common major diseases. Sixteen were entirely nontoxic. Six extracts were possibly very mildly toxic, one mouse succumbing to each. One extract, from a cirrhotic liver, killed 3 mice of 15 injected, but other extracts from cirrhotic livers were nontoxic. In all, 299 mice were injected with the 23 control extracts, with only 9 deaths.

Extracts of lungs from 3 cases of nonspecific ulcerative colitis were tested. Two were not toxic in the doses used while the third was very potent. This extract, when retested 15 months later, was still highly toxic indicating that the toxic factor was stable. Fifty-nine control lung extracts were injected into 451 mice. There were no deaths in shock.

In only one case of nonspecific ulcerative colitis were extracts obtained from both the liver and the lungs. Both proved to be nontoxic, accounting for the one nontoxic liver and one of the 2 nontoxic lungs. This result was not surprising because death in this case was not that of typical nonspecific ulcerative colitis. The patient had had an ileostomy 18 months before death with resultant improvement of the colitis, and she died from peritonitis following a plastic operation on the ileostomy, having at that time a clinical remission and only a little ulceration of the colon.

* This investigation was aided by a grant from the Commonwealth Fund.

1 Steiner, Paul E., *Am. J. Path.*, 1941, **17**, 667.

TABLE I.
Toxicity of Extracts of Human Liver and Lung in Cases of Nonspecific Ulcerative Colitis and Controls.

Exper.	Diagnosis	Wt of tissue, g	Wt of extr., g	Amt inj. per mouse, mg	No. of mice inj.	Acute deaths (4 hrs)	% acute (4 hrs)
Liver 4977	Nonspecific ulcerative colitis	1246	5.1	500	10	9	90
" 5734	" " "	1790	11.4	500	9	9	100
" 5734	(Acid-soluble fraction)	—	—	13	8	2	25
" 5734	(Ether- " "	—	—	500	6	6	100
" 5906	Nonspecific ulcerative colitis	1646	6.6	500	6	4	67
" 5906	(Acid-soluble fraction)	—	—	9	6	5	83
" 5906	(Ether- " "	—	—	500	6	2	33
" 5947	Nonspecific ulcerative colitis	745	3.3	500	7	0	0
23 Control livers	Misc. diseases	—	—	500	299	9	3
Lung 5525	Nonspecific ulcerative colitis	528	1.5	250	5	5	100
" 5749	" " "	681	1.7	250	4	0	0
" 5947	" " "	728	0.7	250	3	0	0
59 Control lungs	Misc. diseases	—	—	250	451	0	0
Infant lungs	Neonatal deaths	2052	14.7	250	18	17	94
" "	" "	—	—	125	26	5	19

Studies on the Nature of the Toxic Factor. Attempts were made to determine the nature of the toxic factor. Hypoglycemic shock was ruled out by a normal blood sugar level (110 mg %) in a mouse which had been in severe shock for about 30 minutes (performed by Dr. Martin Goldner). Fat embolism was excluded by histological studies, by the controls, and by the acid extracts to be described.

Attempts were made to separate the shock-producing principle from the crude extracts by chemical methods in 2 experiments. The original nonsaponifiable residues representing 2 toxic extracts (Liver 5734 and Liver 5906) were dissolved in ether, and extracted 5 times with 1 Normal sulfuric acid. The combined extracts in each experiment were neutralized with barium hydroxide, filtered, and the filtrate evaporated to dryness. These extracts gave negative colorimetric tests for histamine,[2] but gave positive colorimetric tests for tyrosine or tyramine. Mice were then injected with these acid extracts, each animal receiving that quantity obtained from 0.5 g of the original toxic residue. The ether-soluble fractions were tested in parallel to determine if any toxicity persisted in them. The tests showed that the toxic principle was now divided; in Liver 5734 about half of it was found in the acid-soluble fraction, and in Liver 5906 over half of it was in this fraction.

Since the acid-soluble fraction contained no histamine according to the method used for testing, but did possibly contain tyramine, this substance was tested in pure form for toxicity in mice. Mice were injected with 0.135 mg of tyramine hydrochloride, which was the amount found in each mouse-dose in the acid-soluble fraction of Liver 5734. This amount was without noticeable effects on the animals, as was a dose 4 times as large (0.54 mg). It seemed from these experiments that while the toxic acid-soluble fractions contained tyramine that this substance was not present in sufficient amounts to account for all of the toxicity. It must be attributed to other toxic amines or to other substances.

Discussion. The toxic factor described in extracts of human liver and lung in cases of nonspecific ulcerative colitis was not found exclusively in this disease. It or a toxin which resembles it closely, was occasionally found in dilute or in mild form in extracts of liver in other diseases. It was not found in extracts from numerous control adult lungs, but 2 different extracts prepared from large amounts of pooled lungs (46 lungs, 2,052 g) from stillborn infants were toxic. In contrast to this observation was the behavior of an extract of pooled livers from stillborn infants, which did not contain the toxic factor under comparable conditions.

The factor was not related to the presence of necrotic tissue in the body. Many of the

[2] Koessler, K. K., and Hanke, M. T., *J. Biol. Chem.*, 1919, **39**, 497.

persons had cancer, and indeed some of the lung extracts which were not toxic were obtained from lungs containing primary carcinomas or tumor metastases.

The possibility that the toxic factor was absorbed from or through the denuded surfaces (ulcers) in the bowel was entertained. This idea was supported by the absence of toxicity in the livers from one case of quiescent nonspecific ulcerative colitis, several cases of ulcerated carcinoma of the colon, and one case of superficial ulcerative colitis believed to have been the result of a gold-sodium-thiosulphate reaction, because in none of these colons were the ulcerations extensive. By this explanation, however, the toxicity found in the infant lungs remains unexplained, if it be assumed that the toxic substances are identical. The same objection applies to regarding this toxic factor as the cause of the ulcers during its excretion through the mucosa.

Summary. A toxic factor was demonstrated in extracts of the liver and lungs in cases of nonspecific ulcerative colitis. A factor with similar toxic properties was present in lesser amounts or in milder form in an occasional control extract of liver, and in lungs of stillborn infants, but not in adult control lungs or infant livers.

14435 P

The Flea *Malaraeus telchinum* a Vector of *P. pestis.*

A. L. BURROUGHS. (Introduced by K. F. Meyer.)

From the George Williams Hooper Foundation, University of California, San Francisco, Calif.

In the course of an extensive survey instituted in an important section of the San Francisco Bay region in order to determine the source of the *P. pestis* demonstrated in pools of fleas, 1,356 rodents (Meadow mice—*Microtus californicus* 952, Deer mouse—*Peromyscus maniculatus* 347, Brown rat—*Rattus norvegicus* 40, and Harvest mice—*Reithrodontomys megalotis* 17) were trapped. Their ectoparasites were collected and adequate samples of fleas, both males and females, were cleared and identified. The species determinations and host data are listed in Table I.

It was found that *Malaraeus telchinum* was by far the most numerous of the fleas found on *Microtus californicus,* and that this flea outnumbered *Nosopsyllus fasciatus* about 2 to 1 on *Rattus norvegicus. Opisodasys nesiotus* was slightly more numerous than *Malaraeus telchinum* on *Peromyscus maniculatus.*

Since *Malaraeus telchinum* was the only species of flea found to parasitize all 3 species of rodents known to be spontaneous hosts for *P. pestis,* it was deemed imperative to undertake transmission experiments. Such experiments were prompted by the observations of Eskey and Haas[1] who noted that 74 *Malaraeus telchinum* fed on a plague-infected guinea pig were apparently unable to transmit *P. pestis* to guinea pigs despite the fact the flea developed blockage.

Wheeler and Douglas[2] using white mice as sources for the infection reported the failure of *Malaraeus telchinum* to transmit *P. pestis* in mass feeding experiments on white mice. In the experiments herewith reported, *Peromyscus maniculatus, Microtus californicus,* and the white mouse were used as a source for the infection. The pools of the fleas so infected were permitted to feed on *Microtus californicus.*

Ten experiments have thus far been completed, and 4 positive transmissions have been obtained as follows:

(1) Seventy-five fleas were fed on a moribund plague-infected *Peromyscus maniculatus.* Six of the fleas were triturated in a mortar

1 Eskey and Haas, *Pub. Health Bull.,* No. 254, 1940, 42.

2 Wheeler and Douglas, PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 65.

TABLE I.

Host	Species of flea
M. californicus	*Catallagia vonbloekeri* (Augustson, 1941) *Atyphloceras multidentatus* (C. Fox, 1909) *Hystrichopsylla gigas dippiei* (Rothschild, 1902) *Malaraeus telchinum* (Rothschild, 1905) *Peromyscopsylla ebrighti* (C. Fox, 1926)
P. maniculatus	*Atyphloceras multidentatus* *Hystrichopsylla gigas dippiei* *Opisodasys nesiotus* (Augustson, 1941) *Malaraeus telchinum* *Catallagia vonbloekeri*
R. norvegicus	*Malaraeus telchinum* *Nosopsyllus fasciatus* (Bosc, 1801) *Orchopeas sexdentatus* (Fox, 1909)
R. megalotis	*Catallagia wymani* (Fox, 1909)

with salt solution, and inoculated into a guinea pig which died on the 8th day with lesions of plague. A *Microtus* was placed in the crock with the remaining 69 fleas. The animal died in 13 days with the anatomical findings and the cultural isolation of *P. pestis*.

(2) One hundred *Malaraeus telchinum* were placed on a *Microtus* with a terminal plague septicemia; the rodent died within a few hours. Three days later a *Microtus* placed in the crock with the fleas contracted plague and died on the 15th day after exposure.

(3) Seventy-five fleas were placed on a white laboratory mouse with many plague bacilli in the blood. Twenty-four hours after the death of the mouse, a *Microtus* was exposed in the crock. Six days later it was found dead from plague.

(4) An identical experiment was conducted with 25 *Malaraeus*. The *Microtus* contracted plague and died on the 6th day after exposure. Experiments to explain the earlier failures and to evaluate the vector efficiency are in progress.

The valuable assistance of Mr. Robert Holdenried in collecting the specimens and in making the specific determinations of the mammals, and of Dr. M. A. Stewart with the flea identifications is gratefully acknowledged.

14436

On the Specificity of Bacterium-Decarboxylase.

E. GEIGER.

From the VanCamp Laboratories, Terminal Island, Calif.

In a recent study[1] on the histamine-formation in fish muscle it was found that a minor fraction of histamine-units produced post mortem by bacterial action remains within a protein complex and can be liberated only by acid hydrolysis or by fermentative proteolysis. It was assumed, therefore, that histidine groups need not be present in a free form in order to be converted into histamine groups. This implies that some bacterial ferments are able to split such peptide-linkages which involve the carboxyl group of histidine groups without attacking simultaneously those linkages by which the histamine group is attached to the protein molecule. The possible formation of such compounds was discussed by Guggenheim[2] who assumed on a theoretical

[1] Geiger, E., Courtney, G., and Schnakenberg, *Arch. Biochem.*, in press.

[2] Guggenheim, M., *Biochem. Z.*, 1913, **51**, 369.

basis that "bacterial or fermentative processes which produce amines from aminoacids may also convert polypeptides into peptamines." In view of some recent experiments by Gale[3] in which a high specificity of bacterial decarboxylases was demonstrated, it was desirable to investigate the possibility of bacterial decarboxylation of some histidine derivatives. Since the hydrolytic action of bacterial peptidases coincides with the decarboxylation of di- and polypeptides it was investigated first whether histidine derivatives containing monoacylated aminogroups like benzoyl- or acetylhistidine can be decarboxylated by bacteria.

Methods. The benzoylhistidine (1α-benzoylamino-β-imidazolyl-4(5)-propionic acid) and benzoylhistamine (N-β-imidazolyl-4(5)-ethyl-benzamid) were prepared according to Gerngross,[4] and had a melting point 247° and 148°C (cor) respectively. Our monoacetyl histidine sample was obtained according to Bergmann and Zervas[5] (m.p. 169°C cor.). Acetyl-histamine was prepared from free histamine using the same method; a pale yellow solid was obtained, which until now did not crystallize. The decarboxylation was performed according to Gale,[3] however lower concentrations of the substrate were added than in Gale's experiments since benzoylhistidine is less soluble at acid reaction than histidine. Each set of experiments was performed with 3 different strains of *Escherichia coli,* cultured from human feces. It was shown in control experiments that all 3 strains were strong histamine-formers, viz., they decarboxylated added histidine almost quantitatively. A culture of *E. coli* in 500 ml of tryptose broth (pH 5.0) was incubated at 30°C for 16 hours, centrifuged and washed twice with sterile saline solution. The bacteria were then suspended in 10 ml of the saline. To this suspension we added 10 ml of phthalate-buffer (pH 5.0) and 50 mg of benzoylhistidine or acetylhistidine in 30 ml of water. A blank was prepared in the same way but contained 10 ml of water instead of the bacterial suspension. The samples were incubated for 6 to 12 hours at 37°C. The biological activity was assayed on the isolated guinea pig intestine, suspended in 20 ml of Locke-Ringer solution.

Results. After incubating acetyl- or benzoylhistidine with the bacteria even for 12 hours, the ninhydrin reaction, which would indicate the presence of free histidine, remained negative. Consequently *E. coli contains no acylases which would be able to split acetyl- or benzoylhistidine.* The mentioned reaction became however strongly positive after hydrolysing the sample with 5% HCl for one hour.

It was then investigated whether or not acylated histamines can be produced by *E. coli* from acetyl- or benzoylhistidine by decarboxylation. After 12 hours incubation no histaminelike activity on the guinea pig ileum was detected. The same intestinal strip responded to 0.05-0.1 γ of histamine. The failure to contract could be attributed either to the inability of the bacteria to decarboxylate the acyl compounds or to the pharmacological inactivity of the decarboxylated compounds. Therefore the action of benzoyl- and acetylhistamine was studied and it was found that they were without effect even in concentrations which were 100,000 times higher than the minimum effective histamine-concentration. After hydrolysing the solution of benzoyl- or acetylhistamine with 10% HCl on a boiling waterbath for one hour the solution had the expected very strong action on the intestine due to the formation of the free base.

On the basis of these experiments it seemed possible that during the incubation with bacteria the acylated histidines were decarboxylated to give biologically inactive acylhistamines. The validity of this assumption was tested by subjecting the incubated solution to acid hydrolysis, after which however no biological activity was found. Such a hydrolysis would liberate active histamine from its inactive acetyl- or benzoyl-derivatives. We claim therefore *that during the incubation with bacteria no benzoyl- or acetyl-*

3 Gale, F., *Biochem. J.*, 1940, **34**, 393; 1941, **35**, 67.

4 Gerngross, O., *Z. f. physiol. Chem.*, 1920, **108**, 50.

5 Bergmann, M., and Zervas, L., *Biochem. Z.*, 1928, **280**, 203.

histamine was produced from the corresponding histidine derivatives.

That the bacterial decarboxylase is not inhibited by acyl-histidines was shown by the following experiments:

Sample A: Bacterial suspension + 50 mg histidine.

Sample B: Bacterial suspension + 50 mg histidine + 50 mg acetylhistidine.

Sample C: Bacterial suspension + 50 mg acetylhistidine.

After 12 hours incubation only Sample C had no activity on the intestine, while Samples A and B contained equal amounts of histamine (35-40 mg), indicating that presence of acetylhistidine does not inhibit the decarboxylation of histidine. Analogous results were obtained with benzoylhistidine.

Summary. The reported experiments show that the ability of *E. coli* to decarboxylate histidine and its derivatives is highly specific, since the acylation of the amino group hinders this process. We cannot decide however whether or not the *E. coli* are able to convert peptides into "peptamines" by decarboxylation. If a free amino group should be necessary also in peptides, then such amino group could be provided not only by histidine but also by peptides containing histidine units with free carboxyl groups.

We are indebted to Dr. C. Niemann (California Institute of Technology) for valuable suggestions.

14437

Effect of Low Potassium Diet and Desoxycorticosterone on the Rat Heart.*

DANIEL C. DARROW. (Introduced by Grover F. Powers.)

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In a previous publication[1] attention was directed to lesions produced in the heart by injections of desoxycorticosterone acetate. This was to be expected since heart lesions develop on diets low in potassium[2,3,4] and injections of desoxycorticosterone acetate produce a deficit of potassium and changes in muscle composition similar to those accompanying diets low in potassium.[1,5,6] The lesions in the heart are not necessarily accompanied by changes in the electrolyte composition of the heart, and in rats it has not been certain that loss of heart potassium is produced either by diets low in potassium or by injections of desoxycorticosterone acetate. Study of the effects of the combination of a diet low in potassium together with injection of desoxycorticosterone acetate was, therefore, undertaken to determine if heart potassium can be reduced in rats and if lesions are more quickly produced by this means.

The chemical methods and the low potassium diet were described in previous studies.[7] Owing to the size, the hearts of several animals were pooled and analyzed as a whole. Sufficient material was not available to include fat and chloride analyses. All values are expressed per 100 g of dried tissue.

Table I shows the results. While neither the diet low in potassium nor injections of desoxycorticosterone give more than a questionable lowering of heart potassium in 30 days, the combination of the diet low in potassium

* Aided in part by a grant from the Fluid Research Fund.

1 Darrow, D. C., and Miller, H. C., *J. Clin. Invest.*, 1942, **21**, 601.

2 Schrader, G. A., Pricket, C. O., and Salmon, W. D., *J. Nutrition*, 1937, **14**, 85.

3 Follis, R. H., Oerent-Keiles, E., and McCollum, E. V., *Am. J. Path.*, 1942, **18**, 29.

4 Thomas, R. M., Mylon, E., and Winternitz, M. C., *Yale J. Biol. and Med.*, 1940, **12**, 345.

5 Ferrebee, J. W., Parker, D., Carnes, W. H., Gerity, M. K., Atchley, D. W., and Loeb, R. F., *Am. J. Physiol.*, 1941, **135**, 230.

6 Heppel, L. A., *Am. J. Physiol.*, 1939, **127**, 385.

7 Miller, H. C., and Darrow, D. C., *Am. J. Physiol.*, 1940, **130**, 747.

TABLE I.
Heart: Content per 100 g of Dried Weight.

	No. of rats	Experimental, days	DOCA, mg/day	Water, g	Na mM	K mM	P mM
Control	6	0	0	334	17	37	31
DOCA	6	30	2	355	19	35	29
L.K.	6	30	0	359	15	35	31
L.K. + DOCA	6	4	2	355	21	32	31

DOCA rats received 2 mg of desoxycorticosterone acetate daily for 30 days.
L.K. rats received the diet low in potassium for 30 days.
L.K. + DOCA rats received both the low potassium diet and desoxycorticosterone acetate for 4 days.

and the injections of desoxycorticosterone acetate produce significant lowering of heart potassium in 4 days. It will be noted that heart sodium is raised significantly. The data do not demonstrate that the increase in sodium represents an intracellular replacement of the lost potassium by sodium since the small amount of tissue did not permit chloride analyses. However, such a replacement is almost certainly indicated since heart water is the same in this group as in the other experimental groups and total water would be increased in relation to tissue solids if extracellular sodium were appreciably increased.

In parallel experiments lasting a week, the hearts were saved for histological examination. Lesions consisting of necrosis of the myocardium and cellular infiltration were found in 4 of 6 rats. Since neither the diet low in potassium nor injection of desoxycorticosterone acetate produced lesions regularly before 20 days, the combination accelerates the production of lesions as well as brings out the loss of heart potassium.

These findings may have some clinical significance since the administration of large amounts of desoxycorticosterone acetate to a patient receiving a diet low in potassium or large infusions of glucose solutions may lead to loss of muscle potassium. Indeed, certain data[8] show that continuous infusions of salt solution alone lead to deficits of potassium.

Summary. A diet low in potassium when accompanied by injections of desoxycorticosterone acetate produces in 4 days loss of potassium and gain in sodium in the rat heart. Lesions are induced within a week by this treatment.

[8] Stewart, J. D., and Rourke, M. G., *J. Clin. Invest.*, 1942, **21**, 197.

14438

Role of Inositol in Alopecia of Rats Fed Sulfasuxidine.

EDWARD NIELSEN AND A. BLACK. (Introduced by G. A. Harrop.)
From the Biological Laboratories of E. R. Squibb & Sons, New Brunswick, N.J.

Nielsen and Elvehjem[1] reported that feeding of sulfasuxidine* to rats maintained on a synthetic diet resulted in a biotin and folic acid deficiency. This finding was confirmed by Welch and Wright,[2] Martin,[3] and Totter and Day.[4] The present report concerns the effectiveness of inositol in the pre-

[1] Nielsen, E., and Elvehjem, C. A., *J. Biol. Chem.*, 1942, **145**, 713.

* Sulfasuxidine (succinyl-sulfathiazole) was kindly supplied by Dr. W. A. Feirer of Sharp and Dohme, Glenolden, Penn.

[2] Welch, A. C., and Wright, L. D., *J. Nutrition*, 1943, **25**, 555.

[3] Martin, G. J., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 353.

[4] Totter, J. R., and Day, P. L., *J. Biol. Chem.*, 1943, **147**, 257.

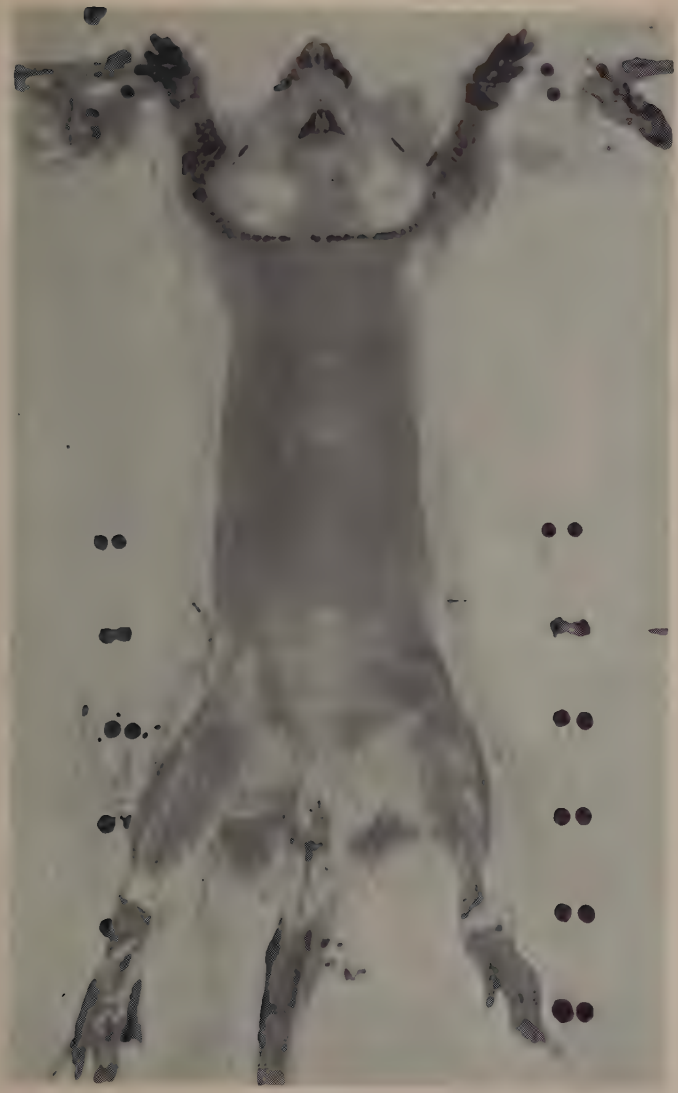

FIG. 1.

vention of alopecia in rats maintained on a synthetic diet containing a sulfonamide.

Experimental. The unmodified basal ration had the following percentage composition: sucrose 73, casein S.M.A. 18, salts IV 4, and corn oil 5. Choline hydrochloride was added to this basal diet at 1 g per kg of ration, while 2-methyl-1,4 naphthoquinone was added at 10 mg per kg of ration. The B-complex vitamins were fed 6 days a week in supplement dishes at the following levels: thiamine hydrochloride 20 γ, riboflavin 40 γ, pyridoxine 25 γ and calcium pantothenate 200 γ. Two drops of halibut liver oil were given each rat per week.

Albino and piebald rats from our colony were used throughout all the experiments. The litters were reduced to 7 animals and only the 1st, 2nd, and 3rd litters were used. The initial weights were 30-40 g and the average

TABLE I.
Incidence of Alopecia.

No. of rats	Modification of ration	Wt gain 8 wks	Incidence
18	Ration 1% sulfasuxidine	40-50 (16 dead)	4
24	" " " + folic acid + biotin	165	11
24	Purified 1% " " " " " " "	140	20
36	" " " " " " " " + 5 mg inositol	174	1

age 19 days. Rats maintained on the above diet grew well and did not develop any symptoms if left on the ration for 15 weeks.

The experimental work was carried out in the following manner. One percent of the sucrose in the above ration was replaced by sulfasuxidine. In this series several deaths resulted after the animals were kept on this regimen for periods longer than 4 weeks. In the surviving animals a low percentage of alopecia was noticed after 6-8 weeks on experiment. In the next series the rats were given daily the equivalent of .5 g of superfiltrol eluate (eluate factor or folic acid) prepared according to the procedure of Hutchings *et al.*,[5] in their supplement dishes together with the other B vitamins. Biotin was injected intraperitoneally at .25 γ on alternate days. Rats receiving this additional supplementation grew very well and after 6 weeks about 40% of the animals showed marked symmetrical alopecia on the belly and on the hind quarters. The symmetrical alopecia is shown in Fig. 1. Slight loss of hair was noticed in a few animals as early as 2 or 4 weeks on this diet.

In the third series the sucrose and casein used in the ration was purified by refluxing with boiling alcohol for 24-48 hours. In one group of 12 rats alopecia was observed in all animals after remaining on the diet 8 weeks. In another group of 12 rats, 8 animals developed marked alopecia. The purification of the sucrose and casein made it possible to produce alopecia in at least 75% of the rats.

[5] Hutchings, B. L., Bohonas, N., and Peterson, W. H., *J. Biol. Chem.*, 1941, **141**, 521.

Since Woolley[6] has shown that inositol is required in mouse nutrition to prevent alopecia, it was suggested that inositol should be investigated. To date we have prevented the alopecia in 36 rats with one exception by feeding in supplement dishes 5 mg of inositol 6 days a week. After definite alopecia had developed 5 mg of inositol was fed daily and definite improvement was noted in several animals after 8 weeks of therapy. In the rats receiving inositol as a preventative measure the fur coat was normal and the luster of the hair was comparable to animals kept on a complete stock diet. The growth over a 9-week period was greater in the male rats receiving inositol as compared to the unsupplemented animals. Those receiving inositol gained on an average 202 g while the negative control animals gained 169 g over the same period. There was no marked difference in the growth of the females.

During this investigation the following diets were tried. The complete replacement of the sucrose by lactose with and without the drug offered no advantage and many early deaths and kidney damage were observed The casein in the diet was increased to 60% by replacing 42% of the sucrose and no significant differences were noted.

Summary. The inclusion of sulfasuxidine in a synthetic diet allows the development of a symmetrical alopecia in rats. Inositol supplementation prevents the onset of this condition. Animals receiving inositol grow better and have a more tidy appearance.

[6] Woolley, D. W., *J. Biol. Chem.*, 1941, **139**, 29.

14439

Momentary Atrial Electrical Axes in Paroxysmal Tachycardia.

GEORGE DECHERD, ARTHUR RUSKIN, AND GEORGE HERRMANN.

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A recent study[1] of our cases of paroxysmal atrial tachycardia has shown the presence of some grade of A-V block with dropped ventricular beats in a large proportion of the group. The block varied in duration as well as degree, and seemed most likely to appear in patients who had either severe myocardial disease or digitalis over-dosage. This led us to the observation,[2] which has also been reported by Barker and his associates,[3] that this group of patients was prone to develop atrial flutter or fibrillation, or both, in close association with the episodes of paroxysmal tachycardia with block. These facts logically raise the question of a possible resemblance between the fundamental disturbances of atrial mechanism in these 3 types of arrhythmia. Barker *et al.*[4] have suggested, in fact, that a circus movement is probably present in each instance. Others have made similar proposals, which have been reviewed elsewhere.[2,5]

Lewis, after preliminary animal experiments, reported studies of 3 patients,[6] one who had flutter and 2 who had fibrillation. Using simultaneous chest leads placed in 3 planes, he was able to calculate the direction of movement of the atrial electrical axis for each 1/50 second. His results are compatible with the presence of a circus movement in flutter and fibrillation, though they can scarcely be said to supply conclusive proof. One possible approach to the above problem seemed to be the analysis of similar tracings, taken in patients with paroxysmal atrial tachycardia. It is fortunate that the electrocardiograms of the patients most likely to show flutter or fibrillation, *i.e.*, those with A-V block during the paroxysm, are best suited for this type of analysis; while the block is present, at least half of the P waves will avoid superimposition on the preceding T wave.

Method. Accordingly, Lewis' technic was repeated in a series of patients showing both normal and abnormal atrial mechanisms. For the sagittal plane, the right arm electrode was placed on the manubrium sterni, the left arm electrode below the xiphoid, and the left leg electrode just to the right of the 7th dorsal spine. For the horizontal plane the right arm electrode was placed below the left nipple, the left arm electrode below the right nipple, and the left leg electrode left in position in the back. For the frontal plane, the left leg electrode was shifted to the manubrial position. In each plane the electrodes were placed as nearly equidistant as possible. It must be borne in mind that these planes are only approximately vertical to one another. The sagittal plane deviates slightly to the right posteriorly, and the horizontal plane slants downward to a variable degree. Using the Sanborn Tri-Beam, two leads may be taken simultaneously, and each pair taken in sequence. The simultaneous chest leads, taken in 3 planes, were enlarged 10 diameters by an opaque projector, and traced onto graph paper. The magnitudes of potentials of the P waves were measured from the generally accepted zero line of the T-P interval. The vectors of the electrical axes were calculated from Leads I and III for each plane, using Einthoven's triangle, every 1/100 second. We have considered it desirable to use a method of graphing the momentary elec-

1 Decherd, G. M., Herrmann, G. R., and Schwab, E. H., *Am. Heart J.*, 1943, **26**, 446.

2 Decherd, G. M., and Herrmann, G. R., in press.

3 Barker, P. S., Wilson, F. N., Johnston, F. D., and Wishart, S. W., *Am. Heart J.*, 1943, **25**, 765.

4 Barker, P. S., Johnston, F. D., and Wilson, F. N., *Am. Heart J.*, 1943, **25**, 799.

5 Barker, P. S., Wilson, F. N., and Johnston, F. D., *Am. Heart J.*, 1943, **26**, 435.

6 Lewis, T., Drury, A. N., and Iliescu, C. C., *Heart*, 1921, **8**, 341, 361.

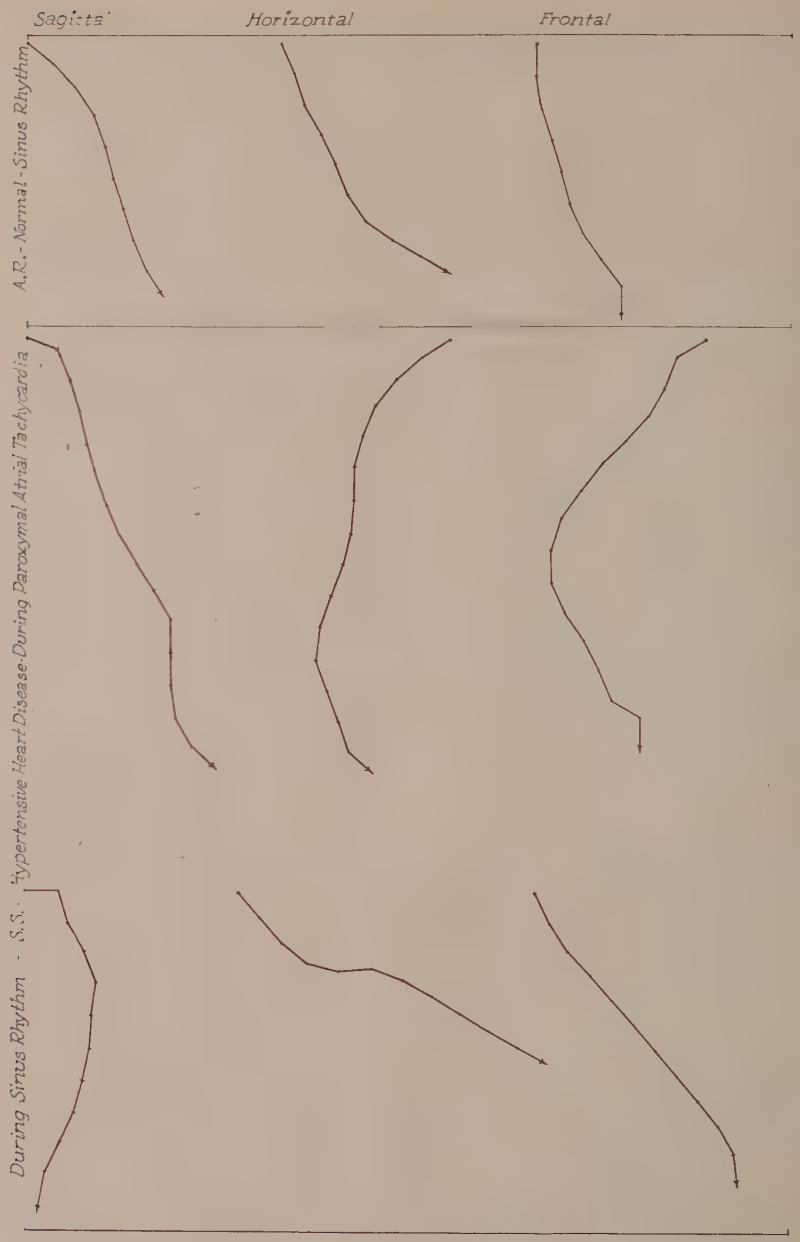

FIG. 1.

Curves showing the consecutive atrial electrical axes; each segment represents the axis for 1/100 second. The sagittal plane is viewed from the right, with the subject erect; the horizontal plane is viewed from above, with the back of the subject at the top of the diagram; the frontal plane is viewed from the front, with the patient erect.

The upper set of curves are a typical normal sinus rhythm.

The middle set is derived from a paroxysm of atrial tachycardia; the lower set is from the same patient, taken during sinus rhythm.

trical axes which differs from those employed by Lewis. A line of unit length has been drawn for the first 1/100 second at the calculated angle from a zero-point; a line of similar unit length for each 1/100 second was then drawn, at the proper angle, using as its zero-point the end of the preceding line. In this fashion is derived a curved line, made up of 1/100 second segments, which shows the direction of change of the electrical axes. Although the curve thus obtained rests ultimately on an anatomical basis, the relationship is an indirect one. It is merely a series of vectors showing only time and direction, and not magnitude, of the electrical potentials.

Though this method is obviously subject to some error, from both the theoretical and practical standpoints, the results obtained have been surprisingly satisfactory. It is well recognized that the hypothesis embodied in the Einthoven triangle is subject to serious error. The triangle is not perfectly equilateral, the heart is not placed in the center thereof, and the electrical properties of the adjacent tissues do not render them a homogeneous conducting medium. Schellong[7] has reviewed these objections, as well as the empirical usefulness of the triangle. In view of the many sources of error, the curves obtained in the 3 planes show remarkable agreement, and permit visualization in 3 dimensions.

Results. In normal individuals with sinus rhythm the curves representing consecutive electrical vectors pass, with some individual variation, downward, forward, and to the left. While there have been considerable individual variations in direction and amount of curvature, due possibly to differences in habitus and position of the atria, the general trend has been uniform. A fairly typical set of normal curves is shown in Fig. 1. In 2 patients with dextrocardia, one with situs inversus, and one with extensive cystic disease of the left lung, the curves have moved downward, forward, and to the right. The variations due to heart disease, with atrial fibrosis or enlargement, will be discussed elsewhere.

We have had the opportunity thus far to study one patient with flutter, and a large number with atrial fibrillation. The data obtained appear to be in fairly good agreement with those reported by Lewis, in that the consecutive axes inscribe a curve which in some instances closely approximates a circle or oval, and in other cases shows different degrees of deviation from this theoretical ideal. We will not discuss at this time the question of whether or not there is a circus movement in flutter and fibrillation, but content ourselves with the statement that if it does exist, this method seems capable of showing it, in our hands as well as Lewis'.

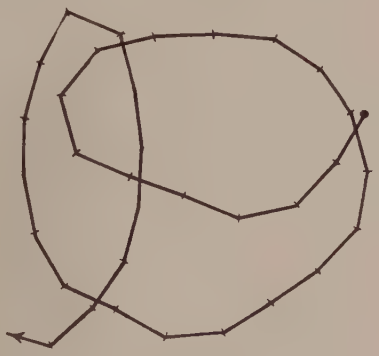

FIG. 2.
Horizontal plane; atrial fibrillation. Consecutive area for 34/100 second.

Electrocardiograms have been analyzed from 6 patients with paroxysmal atrial tachycardia. The projections into the 3 planes employed have been illustrated for one case in Fig. 1. In this instance the curve passes downward, forward, and to the right with a convexity towards the right. The atrial axes in the same patient, taken during sinus rhythm, are shown just below in the same figure; they are well within the normal range, *i.e.*, downward, forward, and to the left. Two other cases of paroxysmal tachycardia have shown the same general direction of the curves representing the consecutive electrical axes. In 2 cases the curves have been directed downward and to the right, but backward instead of forward. The sixth of the group thus far examined showed a curve which ran upward, backward, and to the left, with a left anterior convexity.

[7] Schellong, F., *Erg. inn. Med.*, 1939, **56**, 657.

These curves show varying degrees of curvature; however, so do many of the curves from other patients with heart disease, but with sinus rhythm. In no instance does the curve obtained in paroxysmal tachycardia show the tendency to inscribe a circle or oval, such as has been seen in flutter and fibrillation. For purposes of comparison, Fig. 2 illustrates the axes for 34 consecutive 1/100 seconds, taken in the horizontal plane, from a patient with mitral stenosis and atrial fibrillation; they inscribe a varying circus movement, clockwise in direction.

Comment. Further study of similar cases must be made when opportunity offers, but the contours of the curves of the momentary electrical axes in these cases of paroxysmal tachycardia resemble those obtained in normal sinus rhythm; only their direction is different. On the basis of our present experience, and using a method which appears capable of showing a circus movement if it be present, we are forced to conclude that our evidence points to the presence of an ectopic pacemaker, rather than a circus movement, in paroxysmal tachycardia.

14440

The Excystation, Cultivation and Encystation of *Endamoeba coli.*

M. FRANCES MAYFIELD. (Introduced by E. C. Faust.)

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Although much experimental work has been done on the cultivation of the intestinal amebæ, most of the time and effort has been spent on the pathogen, *Endamœba histolytica.* This organism has been cultured *in vitro* for two decades, but the closely related organism, *Endamœba coli,* has presented difficulties in cultivation. Many protozoologists have found that the latter species tends to die out after a few transfers and have concluded that it cannot be maintained in culture indefinitely. Dobell[1] says, *Endamœba coli* is indeed more difficult to cultivate properly than any other amœba living in man." From the present study it has been found that in the majority of cases cysts of *E. coli* from man will excyst and reproduce *in vitro* and can be maintained in culture for many months. The original strain which was planted from cysts in March, 1942, is still in cultivation after a period of 19 months and shows no indication that it will not continue to live and reproduce in the test tube.

Twenty-six strains of *Endamœba coli* were used for this series of culture experiments. Two were in the trophic and 24 in the cystic stage. All were from human material and none had any associated amebæ. All diagnoses were checked by members of the Department of Tropical Medicine, Tulane University. Examinations were made by the use of unstained, iodine-stained, and iron-hematoxylin-stained preparations.

Method Used to Obtain Maximum Number of Organisms for Culture. In order to obtain a maximum yield of cysts from a stool specimen, a modified combination of the zinc sulphate centrifugal floatation method (Faust *et al.*[2]) was used with the Lane[3] superimposed cover-glass technic. The specimen was thoroughly emulsified with tap water, 10 cc portions were strained through cheese cloth into Wassermann tubes and centrifugalized for 70 seconds at 2600 r.p.m. The supernatant fluid was poured off the tubes, the packed sediment broken up, washed and centrifugalized again. This process was repeated until the supernatant fluid became clear. Zinc sulphate of the specific gravity 1.180 was then added to

1 Dobell, C., *Parasitol.*, 1936, **28**, 541.

2 Faust, E. C., *et al.*, *Am. J. Trop. Med.*, 1938, **18**, 169.

3 Lane, C., *Trans. R. Soc. Trop. Med. and Hyg.*, 1924, **18**, 278.

the brim. A square 22 mm cover glass was superimposed on the full tube and held in place by 4 metal fingers attached to the metal tube. The preparation was recentrifugalized, the cover-glass removed rapidly but carefully and the material which had collected thereon was washed off into a container with a small amount of physiological salt solution. After the cysts had been collected, they were washed 6 times in physiological salt solution in an effort to remove the zinc sulphate and the associated bacteria.

Excystation. Seven combinations of culture media were used at the beginning of the problem. Five of these utilized as a base coagulated whole egg slant; one, coagulated egg albumin; and the other, inspissated human serum. Various liquid overlays were tested, including Locke's serum solution, plain Locke's solution, Ringer's (without sugar) and Ringer's serum solution, egg-albumin Ringer's solution and liver extract broth, also a small quantity of starch. Locke's egg-serum medium plus starch was the only one in which excystation occurred. The initial pH value of this medium was 7.8-8.0 and the final pH was 6.8-7.0. The optimum temperature proved to be 37°C. In most cases the amebæ excysted within 72 hours, although in some instances questionable excystation was observed within 48 hours. Of the 24 strains of *Endamœba coli* that were tested, 15 excysted and were maintained in culture for varying lengths of time from 4 to 19 months. Four of these strains are still in culture. No strain that was started from trophozoites grew in subculture.

The failure of some of the strains to excyst in culture was thought to be due to one or more of several factors, namely, the freshness of the specimen used, the size of the cysts, the degree of the infection, the particular bacteria present or the occurrence of supernucleated cysts. It was found that excystation occurred most readily when the cysts were planted from 1 to 5 days after they were obtained from a freshly passed stool. The extremely large, including supernucleated, cysts would not excyst. The heavier the inoculum the greater was the chance of a positive culture. Finally, certain bacteria presumably must be present in the inoculum before excysted amebæ can be recovered.

Excystation in culture, as observed in iron-hematoxylin preparations, consisted of the massing of the protoplasm around the nuclei, and a general loss of staining capacity of the cyst. The multinucleated metacystic ameba which escaped from the cyst wall was a large trophic ameba containing 5 or more nuclei, some of which were apparently degenerate. The cytoplasm was still massed around the nuclei and one by one the amebulæ were split away from the metacyst. These amebulæ each contained a large nucleus with an eccentric karyosome and heavy plaques of chromatin at the periphery. A small amount of cytoplasm surrounded the nucleus. Starch granules and bacteria were readily ingested and mature trophozoites soon developed.

Cultivation. For maintaining cultures over long periods of time Locke's egg serum plus starch proved to be optimum. However, after excystation occurred the trophozoites were maintained for short periods of time in media utilizing the whole egg base and various liquid overlays such as plain Locke's solution, Ringer's solution with sugar, and 0.5% solution of liver extract plus sterile rice starch.

The optimum time for subculturing was 72 hours but it was not detrimental to prolong the time to 4 or 5 days. In some cases cultures were found positive after 10 days. The pH for optimum growth was found to be 7.0 to 7.4 at an incubation temperature of 37°C.

The technic for transfer of the cultures was a precise one. Utmost care was used to prevent the introduction of extraneous bacteria. To guard against such contamination, media and pipettes were sterilized before being used. Capillary pipettes were found to be very satisfactory for the transfer of positive material. The amebæ grow in the deposit of starch in the little pocket formed at the butt of the slanted egg base. In transferring, it is this material that is carried from one tube to the other.

When large numbers of organisms were needed the technic of cultivation was slightly altered. Erlenmeyer flasks of 125 and 250 cc volumes were used. The egg medium was poured into the flasks to a depth of one-half

of the height of the flask and to this 6 loopfuls of sterile rice starch were added. After having been well shaken the entire supernatant fluid from a positive 72-hour culture tube was added to a prepared 125-cc flask. After 72 hours most of the supernatant fluid from this flask was decanted off, leaving only a small amount of liquid and all of the sediment which lay on the solid egg base. Amebæ were to be found growing abundantly in this sediment, which was then well mixed and pipetted into the prepared 250-cc flask and incubated for 72 hours. This method resulted in the development of masses of amebæ which grow vigorously and abundantly.

The cultured trophozoites which have been studied had large nuclei each with a large eccentric karyosome and a nuclear membrane heavy with plaques of chromatin. The pseudopods were typically those of *Endamœba coli:* they were usually blunt, rounded, and granular, at no time hyaline. The cytoplasm was heavily granular, with many bacteria and starch inclusions, was often distinctly vacuolated and exhibited a very sluggish movement.

Encystation. Encystation in culture occurred 4 times spontaneously and was not produced at will. The medium in 3 of these instances was Locke's egg serum plus starch. In the fourth instance encystation occurred in cultures growing in a medium consisting of an egg base with a liquid overlay of a saline extract of the mucosa of the large bowel of man. This medium contained no starch. Subsequent experiments using the same media did not result in encystation. The pH value in each successful case was 6.4-6.6.

Working on the assumption that the possible cause of this encystation was the associated bacteria, the writer tried to isolate and identify the organisms present in the tubes where encystation occurred. The only ones found were *Escherichia coli communis* and an, as yet, unidentified species belonging to the *Colon-Aerogenes-Proteus group.* When these organisms, singly or together, were reintroduced into cultures containing *Endamœba coli* trophozoites, encystation did not occur.

Summary. Twenty-six strains of *Endamœba coli* were used for culture experiments, 2 strains in the trophic stage and 24 in the cystic stage. The former did not reproduce in subculture but 15 of the latter excysted and grew abundantly. Four of these are still being cultivated. A modified Locke's egg serum medium plus starch was the only one in which excystation occurred and proved to be the optimum one for continued growth of the organism. The medium was incubated at 37°, had an initial pH of 7.8-8.0 and a final pH of 6.8-7.0. Encystation occurred spontaneously but was never produced at will.

14441

Studies on Guinea Pigs After Repeated Administration of Paraldehyde.

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Cervello[1] introduced paraldehyde as a hypnotic in animals and then on February 13, 1882, he ventured to try four 0.5 g doses of the drug on himself. The drug was introduced as a therapeutic agent by Morselli[2] at the Royal Asylum of Turin and within a few years, it had been employed by clinicians in both Europe and the United States. Paraldehyde gained a rather general use in most branches of medicine and especially in obstetrics and psychiatry.

Because of the wide acceptance of paraldehyde as a safe drug and because of the controversy as to whether it loses its efficacy on

1 Cervello, V., *Arch. per le Scienze Med.*, 1882, **6**, 177.

2 Morselli, Enrico, *Gaz. d. ospital*, 1883, **4**, 28.

continued use in patients,[3–8] we thought that some tolerance experiments on animals would be of interest.

Nineteen normal young adult male guinea pigs, weighing from 418 to 582 g, were used. One volume of the drug was diluted with water to 10 volumes. It was necessary to cool both the solution and the syringe below 18°C to keep the drug in solution. Ten cc of this solution per kilo were injected intraperitoneally, 3 times per week (Tuesday, Thursday, and Saturday). The injections were continued for 4 weeks and the average weight of the animals dropped from 499 to 469 g during this period of treatment.

To study the effects of the paraldehyde on these guinea pigs, the periods of time required for the development of 3 stages in the hypnotic state were used:[9] (1) the time from the injection until the onset of sleep, (2) the time from the injection until the end of sleep, and (3) the time from the injection until the end of hypnosis when an animal could walk about a meter with a steady gait. Each of the animals was observed by the above scheme after each of the 12 injections of the solution of paraldehyde and the data are illustrated in Fig. 1.

Summary. Nineteen adult guinea pigs received three intraperitoneal injections of a 1/10 solution of paraldehyde, 10 cc per kilo, per week for 4 weeks. The average weight of the animals dropped from 499 to 469 g during the series of injections. These animals showed a tolerance to paraldehyde, following repeated administration of large doses of the drug, as indicated by a decrease in both the sleeping time and the length of hypnosis. The average time for the onset of sleep, for each series of injections, varied from about 85 to 120 seconds. The duration of sleep dropped from 95 to 31 minutes and the length of hypnosis dropped from 231 to 167 minutes.

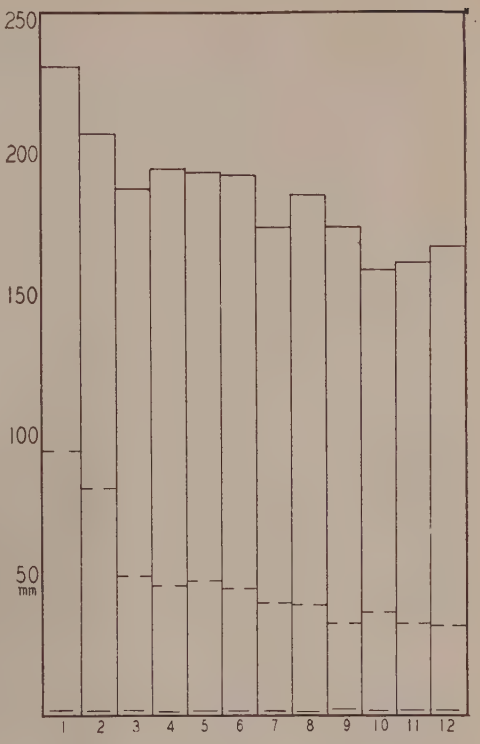

FIG. 1.
Production of Tolerance in Adult Guinea Pigs by 12 Tri-weekly Intraperitoneal Injections of Paraldehyde.

The graphs in Fig. 1 represent averages and each unit shows the results for one day. The lower line represents the time required to produce sleep and the upper broken line represents the end of sleep.

3 Keniston, J. M., *Proc. Connecticut Med. Soc.*, 1888, **4**, 76.

4 Gordon, John, *Brit. Med. J.*, 1889, **1**, 515.

5 MacGregor, Alexander, *The Lancet*, 1899, **1**, 363.

6 Wilson, J. C., *Medical News*, 1883, **43**, 640.

7 Hoyt, F. C., *Medical Record*, 1890, **38**, 520.

8 Mackie, W., *The Lancet*, 1899, **1**, 756.

9 Carmichael, E. B., and Posey, L. C., PROC. SOC. EXP. BIOL. AND MED., 1933, **33**, 1329.

14442

Nicotinic Acid and Pantothenic Acid as Essential Growth Factors for *Shigella paradysenteriae* (Flexner).

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Nicotinic acid is an important and, in many cases, indispensable growth factor for *Shigella paradysenteriæ* (Flexner).[1–3] However, in a medium containing ammonia as the only source of nitrogen, only 4 out of 18 strains were able to grow with the addition of nicotinic acid.[3]

We were able to substantially confirm these observations. Besides, we have found that pantothenic acid is an additional growth factor for a not inconsiderable percentage of strains of the Flexner bacillus.

Except in the case of mass cultures mentioned later on, our experiments were made in test tubes containing a total volume of 5 ml of the following medium:

A	Na_2SO_4	12.5 g	
	$MgCl_2 \cdot 6H_2O$	0.05 g	
	Dextrose	12.5 g	
	NH_4Cl	12.5 g	
	Bidistilled water	ad	1000
B	Nicotinic acid in 5 times the required concentration		
C	KH_2PO_4	2.5 g	
	K_2HPO_4	7.5 g	
	Bidistilled water	ad	1000
D	d-Calcium pantothenate in 5 times the required concentration		

Each solution was sterilized separately and the test tubes filled with 2 ml solution A, 1 ml solution B, and 1 ml solution D. After sterilization, 1 ml of solution C was added under sterile precautions.

The phosphate solution had to be added separately because, with higher concentrations of calcium pantothenate, the formation of a precipitate of calcium phosphate was observed after heating. In the case where nicotinic acid or pantothenic acid were omitted, the solutions B or D were replaced by sterile water.

The medium without growth factors—that is, 2.0 ml "A" + 1.0 ml "C" + 2.0 ml H_2O—is hereafter designated as "basic medium."

All tests were made in duplicate tubes, and where growth was observed, 5 passages in the same medium were made, not counting the initial transfer from broth into the synthetic medium.

The sterility of the media was controlled by incubation of uninoculated tubes for 14 days. All tubes were closed with a Parawax cover over the cotton stopper. Inoculations were made each time with three 3-mm loops of culture.

The experiments were arranged in groups which served as controls for each other; for instance, a group on "basic medium" was put on test simultaneously with one containing nicotinic acid alone, one with pantothenate alone, and one with nicotinic acid and pantothenate. All experiments were repeated at least twice. Before a result of "no growth" was accepted as final, inoculations were repeated 4 to 6 times; thus each such statement is supported by 8 to 12 individual inoculations.

Observation for growth was continued for 14 days at 37°C. Altogether 20 strains were investigated whose cultural and serological properties had been carefully established at the start. The strains were identified anew after they had gone through the 5 passages. About half of the strains were old stock strains which, however, had preserved their smoothness. The other half were strains isolated between November, 1942, and April, 1943.*

Results. In confirmation of previous observations, only one of the 20 strains grew regularly and easily in the "basic medium."

1 Koser, S. A., Dorfman, A., and Saunders, F., Proc. Soc. Exp. Biol. and Med., 1938, **38**, 311.

2 Kligler, I. J., and Grosowitz, N., *J. Bact.*, 1939, **38**, 309.

3 Dorfman, A., Koser, S. A., Reames, H. R., Swingle, K. F., and Saunders, F., *J. Inf. Dis.*, 1939, **65**, 163.

* We wish to thank Drs. A. B. Wadsworth, A. V. Hardy, E. Neter, and O. Costa Mandry for providing strains for the present investigation.

Fifteen additional strains grew in a medium containing nicotinic acid. Nicotinic acid and nicotinamide were found to serve interchangeably. As a rule, nicotinic acid was employed in a concentration of 1 μg/ml. We found that 0.1 μg/ml served as well and that, as a rule, growth was still possible but delayed and much weaker with 0.01 μg/ml. These data agree in their order of magnitude with those of Koser and his co-workers.[1]

There remained 4 strains which we were not able to cultivate in our ammonium-nicotinic acid medium. A systematic search of the factor lacking was made. We started with a liver extract which was known to contain, besides nicotinic acid, the following growth factors: thiamine, biotin, folic acid, pantothenic acid, riboflavin, and pyridoxine. Three out of the 4 strains grew easily if 0.5% of this extract was added to the basic medium. The factors just enumerated were then paired with nicotinic acid in the following concentrations: riboflavin 5 μg, thiamine 10μg, biotin 0.02 μg, pantothenic acid 1 μg, folic acid 0.05 μg/ml. As it is known that certain amino acids have an activity like an essential metabolite, tubes with 2 mg of tryptophane, 0.4 mg adenine sulfate, and 0.4 mg of cystine per ml, respectively, were included in this experiment. All 3 strains that grow in the medium enriched with liver extract, multiplied freely in passages when pantothenic acid was added. No other of the substances enumerated above allowed propagation of these strains.

No difference of effect was seen in concentrations of calcium pantothenate between 200 and 0.02 μg. As a rule, 0.2 μg calcium pantothenate was used in our tests.

It appears, therefore, that pantothenic acid is an essential growth factor for at least some of the Flexner strains.† There is no convincing evidence that pantothenic acid influences the rate or abundance of growth in strains for which this substance is not essential.

There remains one strain ("V Boyd") that we have not been able to grow in any one of our synthetic media.‡

From 5 of our strains, single tubes were obtained from which variants could be derived that, after a delayed start, were able to grow more or less abundantly without nicotinic acid. We were not able to find a case where growth depends on the presence of pantothenic acid but not nicotinic acid. In the few cases where multiplication occurred in media containing pantothenic but not nicotinic acid, closer examination revealed each time that these variants could dispense with the pantothenic acid as well; in other words, they were just variants that are able to dispense with nicotinic acid. The question arises whether strains that are able to grow without nicotinic acid would be able to manufacture nicotinic acid from the basic medium, or whether they were able to dispense entirely with this substance (and presumably coenzyme). Definite limiting concentrations of nicotinic acid exist for growth of strains which need this factor.[3] It had been concluded from this fact that no nicotinic acid is formed by the Flexner bacillus. We have in 4 cases cultivated variants, that had been found to do without nicotinic acid, in 500 ml volumes of the "basic medium" (without nicotinic acid), and examined the culture for nicotinic acid by the method of Snell and Wright.[5]§ In each case nicotinic acid was found in amounts of the order of 10^{-2} μg/ml. It appears, therefore, that at least these variants of the Flexner bacillus do manufacture nicotinic acid.‖

† There is a statement in the literature[4] that for *Sh. dysenteriæ* (Shiga) pantothenic acid is not an essential factor. From the data given here it will be apparent that it is hazardous to base generalized statements of this kind on the experience with one single strain.

[4] Krauskopf, E. J., Snell, E. E., and McCoy, E., *Enzymologia*, 1939, **7**, 327.

‡ Since this was written, Dr. S. H. Hutner has investigated this strain. He finds that uracil and tryptophane are to be supplied in the medium in addition to nicotinic acid.

[5] Snell, E. E., and Wright, L. D., *J. Biol. Chem.*, 1941, **139**, 675.

§ We wish to thank Mrs. A. J. Dornbush for making these determinations.

‖ Since this was submitted for publication, similar findings were reported for 2 strains of the Flexner bacillus.[6]

[6] Koser, J. A., and Wright, M. H., *J. Bact.*, 1943, **42**, 239.

From the study of cultures recovered from synthetic media it could be ascertained that variation from "smooth" to "rough" and the appearance of dwarf forms is not markedly influenced as long as a reasonably abundant growth is maintained. In some cases when growth was scant—as was occasionally observed with variants growing without supplementary nicotinic acid—an increased tendency to develop rough and dwarf forms was noticeable. In this case also, however, individual differences are so great that no fixed rule can be given.

Discussion. The distinction between "not exacting" and "exacting" strains has been originally established on the criterion of the ability or inability of a microörganism to satisfy all nitrogen requirements with ammonia nitrogen. In the case of the Flexner bacillus we can, on the basis of our present knowledge, circumscribe the meaning of the term "exacting" somewhat more closely. We know now that there exist at least 3 grades of minimal growth requirements, namely: 1. growth is possible with ammonium salt alone; 2. nicotinic acid has to be provided; and 3. nicotinic acid and pantothenic acid have to be available in the medium.

The term "exacting" can, therefore, be more exactly defined as inability to synthesize nicotinic acid and—in case 3—pantothenic acid. Grade (2) will possibly have to be subdivided according to whether or not an intermediary substance (amino acid) will enable the microorganisms to synthesize nicotinic acid.[3]

Exceptional deficiencies in synthesizing ability do occur.

Summary. 1. Former observations concerning the role of nicotinic acid as an essential growth factor for the Flexner bacillus have been confirmed. Individual strains vary greatly in their requirements and often are able to manufacture this substance themselves or produce variants that have this ability. 2. For certain strains of the Flexner bacillus, pantothenic acid is a second essential growth factor.

14443

Increased Efficiency of Phenolic Germicides by Addition of Inorganic Salts to Produce Oxidation-Reduction Systems.

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In a previous communication[1] it was shown that inorganic salts of iron, tin, and manganese, when tested individually, exhibited very little or no germicidal activity against *Staphylococcus aureus*. However, when the salts were mixed in certain combinations and proportions to produce oxidation-reduction systems a pronounced germicidal action occurred. The phenomenon was shown to be a function of the positive metallic ions; the negative ions apparently played no part in the reaction.

The addition of an appropriate metallic salt to an inorganic germicide, *e.g.*, ferrous sulfate to mercuric chloride, to produce an oxidation-reduction system, resulted in a greater effectiveness of the latter. The addition of another oxidation-reduction system, such as a mixture of ferrous and ferric sulfates, to the ferrous sulfate-mercuric chloride combination increased still further the efficiency of the mercuric chloride.

The present communication is concerned with the effect of the addition of inorganic metallic salts to the organic phenolic compounds, phenol, cresol, and Hexylresorcinol.

Results from Phenol. An aqueous solu-

1 Guest, Howard L., and Salle, A. J., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 272.

TABLE I.
Effect of Oxidation-Reduction Systems on Germicidal Efficiency of Phenol.

	Germicidal sol.		Killing dil. for *Staphylococcus aureus* in 10 min. at 37°C
(A)	Phenol		1:100
(B)	Phenol	1.25 g	
	$Fe_2(SO_4)_3 \cdot 5H_2O$	3.25 "	
	Glycerin	50 cc	
	Distilled water to make	500 "	1:1800
	Phenol concentration—1:400		
(C)	Phenol	1.25 g	
	$FeCl_3 \cdot 6H_2O$	3.60 "	
	Glycerin	50 cc	
	Distilled water to make	500 "	1:1500
	Phenol concentration—1:400		
(D)	Phenol	1.25 g	
	$FeCl_3 \cdot 6H_2O$	3.60 "	
	$FeCl_2$	1.70 "*	
	$FeCl_3 \cdot 6H_2O$	7.20 "	
	Glycerin	50 cc	
	Distilled water to make	500 "	1:4500
	Phenol concentration—1:400		

*The mixture of ferrous and ferric chlorides represents another oxidation-reduction system.

TABLE II.
Effect of Oxidation-Reduction Systems on Germicidal Efficiency of Cresol.

	Germicidal sol.		Killing dil. for *Staphylococcus aureus* in 10 min. at 37°C
(A)	Cresol		1:300
(B)	Cresol	1.45 g	
	$FeCl_3 \cdot 6H_2O$	3.60 "	
	Distilled water to make	500 cc	1:4000
	Cresol concentration—1:345		
(C)	Cresol	1.45 g	
	$FeCl_3 \cdot 6H_2O$	3.60 "	
	$FeCl_2$	1.70 "*	
	$FeCl_3 \cdot 6H_2O$	7.20 "	
	Distilled water to make	500 cc	1:12,000
	Cresol concentration—1:345		

*The mixture of ferrous and ferric chlorides represents another oxidation-reduction system.

tion of phenol was found to kill *Staphylococcus aureus* in a dilution of 1:100 in 10 minutes at 37°C.* (Table I-A).

The addition of ferric sulfate to the phenol in the proportion of one gram mole of phenol to one-half gram mole (one equivalent) of ferric sulfate, and some glycerin to stabilize the preparation, increased the efficiency of the phenol 18 fold (Table I-B). Maximum germicidal activity was obtained by mixing the phenol and iron salt in the above proportion.

* The Food and Drug Administration method for the evaluation of germicides was followed throughout.

TABLE III.
Effect of Addition of Ferric Chloride on Germicidal Efficiency of Hexylresorcinol.

	Germicidal sol.		Killing dil. for *Staphylococcus aureus* in 10 min. at 37°C
(A)	Hexylresorcinol		1:6000
(B)	Hexylresorcinol	1.0 g	
	$FeCl_3 \cdot 6H_2O$	1.3 "	
	Distilled water to make	1500 cc	1:24,000

In a similar experiment ferric chloride was substituted for the ferric sulfate in the proportion of one gram mole each of phenol and iron salt. In this instance the efficiency of the phenol was increased 15 fold (Table I-C). Maximum germicidal activity was obtained by mixing the phenol and iron salt in the above proportion.

The addition to the solution of phenol and ferric chloride of another oxidation-reduction system, composed of a mixture of ferrous and ferric chlorides in the proportion of one gram mole of ferrous chloride to two gram moles of ferric chloride, increased the efficiency of the phenol from 15 to 45 fold (Table I-D).

Results from Cresol. An aqueous solution of cresol killed *Staphylococcus aureus* in a dilution of 1:300 in 10 minutes at 37°C (Table II-A).

The addition of ferric chloride to the cresol in the proportion of one gram mole of each increased the killing dilution of cresol from 1:300 to 1:4000 or a 13 fold increase in its efficiency (Table II-B). Maximum germicidal activity was obtained by mixing the two compounds in the above proportion.

The addition to the solution of cresol and ferric chloride of another oxidation-reduction system, composed of a mixture of ferrous and ferric chlorides in the proportion of one mole of ferrous chloride to two moles of ferric chloride, increased the killing dilution of cresol from 1:4000 to 1:12,000 or a 40 fold increase in its efficiency (Table II-C).

Results from Hexylresorcinol. An aqueous solution of Hexylresorcinol killed *Staphylococcus aureus* in a dilution of 1:6000 in 10 minutes at 37°C (Table III-A).

The addition of ferric chloride to Hexylresorcinol in the proportion of one gram mole of each increased the killing dilution from 1:6000 to 1:24000 (Table III-B).

Summary. The addition of an appropriate metallic salt (ferric chloride, ferric sulfate) to the organic compounds phenol, cresol, and Hexylresorcinol to produce oxidation-reduction systems resulted in a great increase in the efficiency of the germicides for *Staphylococcus aureus*. The addition of another oxidation-reduction system to phenol and cresol, composed of a mixture of ferrous and ferric chlorides, increased still further the efficiency of the germicides. It was found that maximum efficiency occurred only if the salts and germicides were mixed in certain definite proportions. The phenomenon applies to oxidation-reduction systems composed of organic germicides with inorganic salts as well as to combinations of inorganic metallic salts and should have far reaching application.

14444

Presence of Typhus Antibodies in Commercial Frozen and Dried Complement.

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Frozen and dried complement from commercial sources has been used by this laboratory for the past two years in the performance of the virus and rickettsial[1,2] complement fixation tests. With the exception of an occasional ampule giving a low titer due to a poor vacuum or inadequate drying, at no time did we experience any serious difficulties with this material. However, in a recent series of tests, we have had results which can be best explained on the basis of the presence of specific antibodies in the particular product being used.

The initial observations were as follows: In a test containing a large number of human sera being titrated against both the epidemic and murine antigens, it was noted that in all tubes containing the epidemic antigen there was complete fixation regardless of the dilution of the test sera even when known negative sera were being tested. As there was no parallel abnormal complement fixation in the presence of the murine antigen (the positive and negative controls gave expected results) the immediate suspicion was that the epidemic antigen had for some reason become anticomplementary. Another epidemic antigen was therefore used on the following day but the complement was unchanged. Again all tubes containing epidemic antigen gave complete fixation even with known negative sera while the series with murine antigen behaved as expected. It was obvious that we were not dealing with a chance phenomenon. The first thought, that the antigen had become anticomplementary, was improbable as our epidemic antigens had been recently shown to be free from anticomplementary action in the dilutions used. It seemed unreasonable to assume a sudden change in 2 antigens particularly as other similar antigens (murine) stored in the identical manner were unaltered. It was therefore suspected that a specific fixation was occurring between the complement and the epidemic antigen as opposed to the murine antigen. Furthermore, this lot of complement was employed in psittacosis and lymphocytic choriomeningitis complement fixation tests without any evidence of abnormal fixation. This complement is hereafter referred to as complement lot No. 1.

Complement lot No. 1 was therefore tested as an unknown serum to see whether the presence of antibodies could be detected. It was inactivated at 56°C for 30 minutes and tested in the usual manner against epidemic antigen in the presence of a completely different complement (complement lot No. 2). At the same time this new complement, lot No. 2, was similarly tested as a control. Routine specific control sera were included in the same test. (Table I.)

It was noted that complement lot No. 1 was capable of fixing part of the complement with epidemic antigen at a serum dilution of 1/80 while complement lot No. 2 gave no fixation.

The epidemic antigen (E-26) that was employed in the preliminary test was also titrated at the same time in the presence of the heat inactivated complement lot No. 1 as well as known positive and negative sera. The results in Table II were obtained.

It is obvious from the above tests that the antigen (E-26) was not anticomplementary at 1/10 in saline nor did it give fixation at 1/10 in 10% normal serum. On the other hand when tested against control Epidemic Human No. 7 it gave a 3+ fixation at 1/160 dilution, the same titer for this antigen as observed in previous titrations. In the presence of complement lot No. 1 diluted 1/30 this antigen titered 1/120 indicating again that there was a considerable degree of react-

1 Plotz, H., and Wertman, K., *Science*, 1942, **95**, 441.

2 Plotz, H., *Science*, 1943, **97**, 20.

TABLE I.
Serum Titrations with Epidemic Antigen E-26.*

Sera	Dilutions: 1/10	1/20	1/40	1/80	1/160	1/320	1/640	SC†
Complement No. 1 inactivated	4	4	4	2+	0	0	0	0
Complement No. 2 inactivated	0	0	0	0	0	0	0	0
Epidemic human serum No. 7 (control)	4	4	4	4	4	4	3—	0

Complement No. 2 titration	0.1 cc	0.15	0.2	0.25	0.3	0.4	0.5
1/42 dil. of complement and 0.25 cc E-26 1/80	4	4	2	±	0	0	0

* 0.5 cc of a 1/42 dilution of complement No. 2 was used in the test.
† Serum control 1/10 dilution with no antigen.

TABLE II.
Epidemic Antigen Titration.

Antigen dilution	Complement lot No. 1 inact. 1/30 dil.	Control Ep. H No. 7 1/80 dil.	Control normal 1/10 dil.	Saline control
1/10	4	4	0	0
1/15	4	4	0	0
1/20	4	4	0	0
1/30	4	4	0	0
1/40	4	4	0	0
1/60	4	4	0	0
1/80	4	4	0	0
1/120	3	4	0	0
1/160	1	3	0	0

*Complement lot No. 2 titration	0.1 cc	0.15	0.2	0.25	0.3	0.4	0.5
1/42 dil. (saline)	4	4	1	0	0	0	0

* 0.5 cc of 1/42 dilution of complement lot No. 2 was used in the test.

ing substances in the heat inactivated complement.

To obtain further evidence that complement lot No. 1 contained specific antibodies it was subjected to a mouse neutralization test. Accordingly, mouse toxicity tests were set up using an epidemic and murine toxic substance. It was shown that no antibodies were present in the complement lot No. 1 which were capable of neutralizing the murine toxic substance. The epidemic toxic substance, however, was completely neutralized by a dilution of 1/32 and partially neutralized by a dilution of 1/64 of complement lot No. 1. This indicates clearly that specific epidemic neutralizing antibodies were present in complement lot No. 1.

Subsequently, we have observed this same phenomenon in a lot of complement from a different commercial establishment. In this latter instance, identical qualitative results were obtained. Not only did the inactivated complement fix in the presence of 3 of our epidemic typhus antigens, but also with 2 commerical epidemic typhus antigens. No fixation occurred in the presence of murine antigen. Furthermore, this same complement completely neutralized epidemic toxic substance at a 1/16 dilution. However, in all these tests the titers were approximately one-half those observed with complement lot No. 1.

During 3 years of experience with the complement fixation test in typhus fever, we have never encountered normal guinea pig sera which gave positive tests. The demonstration therefore of a heat stable substance capable of fixing complement in the presence of epidemic rickettsial antigen but not in the presence of murine rickettsial antigen, psittacosis or lymphocytic choriomeningitis antigens is highly indicative that these particular lots of complement were derived from guinea pigs possessing epidemic typhus antibodies. This opinion is further substantiated by the demonstration of specific neutralizing antibodies for epidemic toxic substance.

The possibility that the complement in question originated from guinea pigs that were used in testing epidemic typhus vaccine is admitted by one of the commercial concerns

who furnished the complement. They likewise corroborated our results in finding specific antibodies in the specimen of complement examined. Measures have been taken by the commercial concern to enforce a previous order to destroy all used animals and further to test all lots of frozen and dried complement for the presence of typhus antibodies.

It is obvious from the above that serious errors in complement fixation can be obtained if complement from guinea pigs of unknown origin is used. It is therefore advisable when complement is obtained from unknown origin to test it for the presence of specific antibodies before use.

The neutralization tests were performed by Captain Byron L. Bennett, Sn.C., and Captain Howard L. Hamilton, Sn.C.; the psittacosis and lymphocytic choriomeningitis virus complement fixation tests were performed by First Lt. Joel Warren, Sn.C., of the Division of Virus and Rickettsial Diseases, Army Medical School.

14445

Lipoxidase, a Dehydrogenase.

JOHN P. HUMMEL AND H. A. MATTILL.

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The action of lipoxidase on unsaturated fats has recently been shown[1] to be of a complex nature; coupled with the oxidation of the fat is a loss of associated carotene which first directed attention to the existence of this enzyme. It appears to catalyze autoxidative rancidity, which has long been known to bleach carotene and hasten the oxidative destruction of fat-soluble vitamins.[2] Since autoxidation can be delayed by phenolic inhibitors and by tocopherol, application of these antioxidants offered a possible means of further studying the manner of action of lipoxidase, whether it is merely a catalyst or whether it modifies the course of autoxidation.

The substrate was the ethyl esters of lard fatty acids,[3] 75 mg properly emulsified in 75 cc of a solution comprising 60 cc water, 9 cc of 95% alcohol, 3 cc of acetone, and 3 cc of 0.1 M phosphate buffer at pH 6.5.

Oxygen absorption was measured at 38° in a macro-modification of the Warburg respirometer; when equilibrium conditions were attained, 3 cc of enzyme solution* were added by upsetting a small container within the flask. Iodimetric determinations of peroxide oxygen[4] were made on parallel samples under identical conditions.

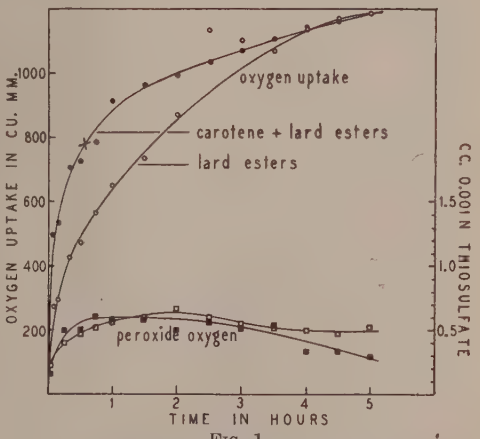

FIG. 1.

Oxygen uptake of esters with ● and without ○ carotene; peroxide oxygen with ■ and without □ carotene. X marks decolorization of carotene.

As seen in Fig. 1, the typical enzymatic

[1] Balls, A. K., Axelrod, B., and Kies, M. W., *J. Biol. Chem.*, 1943, **149**, 491.

[2] Mattill, H. A., *J. Am. Med. Assn.*, 1927, **89**, 1505.

[3] Olcott, H. S., and Mattill, H. A., *J. Am. Chem. Soc.*, 1936, **58**, 2204.

* This crude extract was prepared by suspending 5 g of powdered soybean protein (Glidden flakes, courtesy of Dr. P. L. Julian) in 100 cc of 2% sodium chloride solution. After standing for 30 minutes at room temperature the solution was centrifuged.

[4] French, R. B., Olcott, H. S., and Mattill, H. A., *Ind. Eng. Chem.*, 1935, **27**, 724.

oxidation of lard esters proceeded immediately on a hyperbolic course without a preceding induction period. During this time, there was only a small transient rise in peroxide oxygen.

When carotene was present,† there was a marked increase in the initial oxygen uptake, and this pro-oxidant effect persisted even after the carotene was decolorized. The time of decolorization varied with the amount of carotene present, as short as 1½ minutes in a concentration of 0.1% of the esters, and 90 minutes for 1% concentration. The reported failure of carotene to increase the rate of oxygen absorption[5,6] may be related to the amount of activator present.[1]

Since the simultaneous oxidation of fat is essential to the oxidation of carotene, the simplest explanation of the pro-oxidant effect of carotene is that the active fat peroxides bring about the autocatalytic formation of carotene peroxides which then also initiate new reaction chains, thus favoring the progress of the reaction. The persistence of the pro-oxidant effect of small amounts of carotene subsequent to decolorization might then be due to the additional reaction chains produced during the initial peroxidation.

The peroxide oxygen formed in the early stages of the reaction was small compared to that appearing in normal autoxidation, much too small to account for the oxygen absorbed. The ferrothiocyanate method of Sumner,[7] in our hands, failed to make any distinction between active and inactive peroxides.

In the course of ordinary autoxidation, when peroxides begin to disappear, secondary products make their appearance, thus suggesting that peroxide oxygen is in part used for this purpose. Since peroxide oxygen does not accumulate in the enzyme catalyzed reaction, this oxygen is apparently more promptly utilized; hence the effect of the enzyme on the peroxide oxygen became a matter of interest.

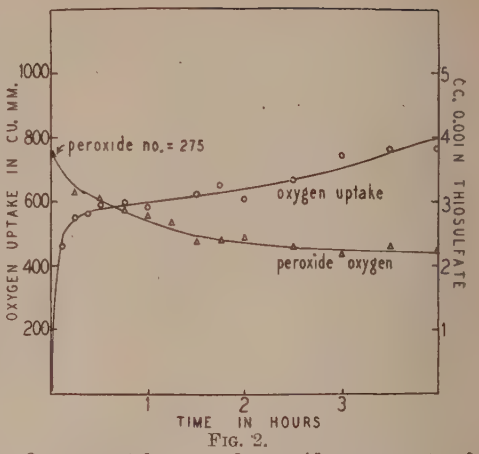

FIG. 2.

Oxygen uptake ○ and peroxide oxygen △ of highly rancid esters.

When rancid esters with a very high peroxide number were used as substrate (Fig. 2), the enzyme produced a marked fall in peroxide oxygen accompanied by a rapid absorption of oxygen only during the first 15 minutes; thereafter, the oxygen uptake was negligible. The only feasible destination of this oxygen was the formation of secondary products. This was confirmed by the presence of carbonyl groups[8] as shown by Schiff's reagent. Thus the enzyme not only accelerates the formation of peroxides, but facilitates their utilization in the creation of secondary products.

Antioxidants when added to the usual autoxidizing fat prolong the induction period. When hydroquinone was added to the enzyme-substrate emulsoid, it did not produce an induction period (Fig. 3); the oxygen uptake rose unimpeded to a level (A) at which it was interrupted until a later time (B) when it rose again to the normal ultimate value. This interruption of the oxygen uptake was noted only with relatively low concentrations of enzyme and was proportional to the amount of hydroquinone added. With higher concentrations of hydroquinone, the initial oxygen uptake proceeded more slowly and to a lower level, at which it remained for the rest of the experimental period.

† 50 mg crystalline carotene (10% α, 90% β) in 200 cc acetone. Desired amounts of this solution were added to the esters before their emulsification in water.

5 Süllmann, H., *Helv. chim. Acta*, 1941, **24**, 1360.

6 Sumner, R. J., *J. Biol. Chem.*, 1942, **146**, 215.

7 Sumner, R. J., *Ind. Eng. Chem., Anal. Ed.*, 1943, **15**, 14.

8 Süllmann, H., *Helv. chim. Acta*, 1942, **25**, 521.

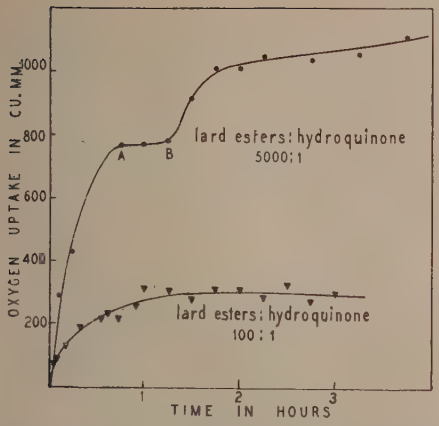

FIG. 3.

Effect of large ▲ and small ● amounts of antioxidant on oxygen uptake.

The initial oxygen uptake in the presence of small amounts of hydroquinone may imply that the enzyme directly attacks certain bonds in the fat molecule without involving the autocatalytic transfer of energy, such that hydroquinone cannot interrupt this initial process. As the reaction proceeds, part of the oxygen uptake may go to the further oxidation of the initially formed peroxides, and part to the formation of further reaction chains inaugurated by these peroxides; hydroquinone may then break these reaction chains.

For information as to the nature of the enzyme, the effects of some common enzyme inhibitors were tried, as well as the anaerobic reduction of a dye. The enzyme has been reported to be relatively uninhibited by cyanide, strongly inhibited by sodium azide and hydrogen sulfide.[5] Using regular Warburg respirometers, we found that sodium sulfide, iodoacetic acid, sodium pyrophosphate, and carbon monoxide markedly inhibited the action of the enzyme.

Two kinds of inhibitors must be taken into account, the true enzyme inhibitors, and the fat antioxidants. The above respiratory inhibitors probably have no antioxygenic action but with some compounds the distinction may not be clear. Of two surface active substances, sodium taurocholate had a very strong inhibitory action, whereas saponin had none.

Most of the respiratory inhibitors used above interfere with the action of the so-called aerobic dehydrogenases. Accordingly, the enzyme was tested (Thunberg tubes) for its ability to reduce a redox dye anaerobically; 2,6-dichlorophenol indophenol was chosen because of its high redox potential and its negligible antioxidant activity. Although some slow decolorization occurred in the absence of substrate, decolorization was very rapid in its presence, thus satisfying one of the most important criteria of an aerobic dehydrogenase. Among the properties which characterize aerobic dehydrogenases are the following: they activate the hydrogen of metabolites; they reduce certain dyes, and act in the absence of oxygen when such dyes are present; they catalyze the direct reaction between metabolites and oxygen, and produce peroxide in the presence of oxygen; they may or may not be inhibited by cyanide, and require neither coenzyme nor cytochrome systems.

The peroxide in this reaction is presumably a fatty acid peroxide rather than hydrogen peroxide which is usually encountered with aerobic dehydrogenases, but the fundamental characteristics of lipoxidase are those of an aerobic dehydrogenase rather than of an oxidase. Oxidases are inhibited by cyanide; this enzyme is not. Oxidases do not produce peroxides and do not decolorize dyes anaerobically. This enzyme does both. Little loss of activity follows dialysis.[5]

The evidence for the dehydrogenase nature of the enzyme may admittedly be criticized because the enzyme preparation was a crude extract; perhaps this contained a mixture of enzymes. If one of them is a dehydrogenase, it could activate the hydrogen of unsaturated fats, possibly by the creation of new double bonds. The answer to this question must await the further purification of the enzyme.

Summary. Lipoxidase appears to catalyze both the primary and secondary phases of fat autoxidation; in the primary phase carotene behaved as a pro-oxidant. Distinction must be made between inhibitors of enzyme action and the accepted inhibitors of the autoxidation of fats. A study of the effects of the former suggests that the enzyme is rather a dehydrogenase unless it is of multiple nature.

14446 P

Recording of Right Heart Pressures in Man.*

A. Cournand, H. D. Lauson, R. A. Bloomfield, E. S. Breed, and E. de F. Baldwin.
(Introduced by W. W. Palmer.)

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Utilizing the right heart catheterization technic of Cournand and Ranges,[1] the cyclic pressure changes in the right auricle and right ventricle have been recorded with the Hamilton manometer[2] in 50 individuals.

Catheterization of the right auricle was performed as previously described.[1,3] The patients experienced little or no discomfort during the procedure. The level of the catheter in the heart by lateral X-ray was taken as zero pressure. Introduction of the catheter with a slightly curved tip into the right ventricle under fluoroscopic control was not difficult as a rule, and was signaled first by a rise above auricular pressure, then by large oscillations at cardiac rate in the saline manometer connected to the catheter.

Addition of the long narrow catheter (No. 8 or 9) to the manometer system decreases the natural frequency. Frequencies obtained with this system have varied from about 25 to 50 vibrations per second. Although not ideal, this range has proved fairly adequate

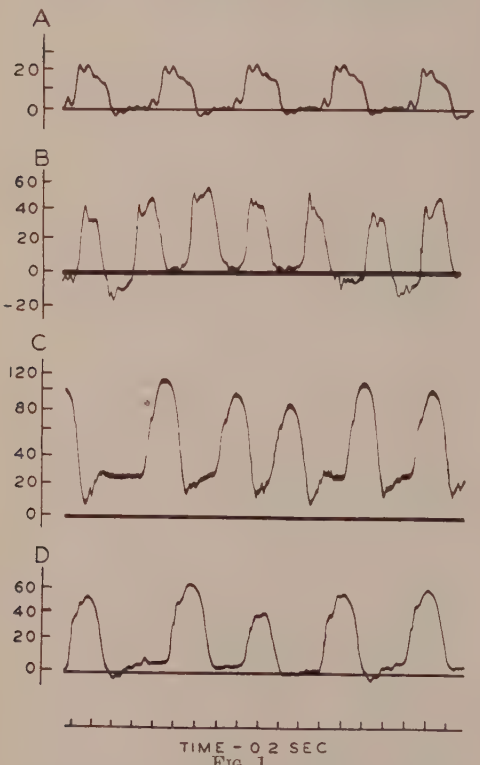

Fig. 1.

All tracings were retouched to improve contrast in reproduction. Pressures in mm of mercury.

A. Right ventricular pressure pulses from a normal young female. One respiratory cycle is shown.

B. Record from young male with extensive pulmonary fibrosis and normal-sized heart. Cardiac output and arterial pressures normal. Note marked increase in systolic pressures; large respiratory variation due to dyspnea at rest; sharp drop of systolic and diastolic levels associated with beginning of inspiration; artifacts on systolic peaks.

C. Patient with mitral stenosis and insufficiency and aortic insufficiency and auricular fibrillation in congestive failure. Note extreme elevation of systolic pressures in right ventricle. The high diastolic pressures correspond to marked increase in right auricular and peripheral venous pressures. Note respiratory variation associated with dyspnea. Cardiac output subnormal and total blood volume about twice normal. Arterial pressure was 210/90.

D. Same patient after clinical improvement due to bed rest and digitalization.

* This investigation was carried on under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Columbia University, with the collaboration of New York University. Additional support was provided by the Commonwealth Fund.

1 Cournand, A., and Ranges, H., Proc. Soc. Exp. Biol. and Med., 1941, **46**, 462.

2 Hamilton, W. F., Brewer, G., and Brotman, I., *Am. J. Physiol.*, 1934, **107**, 427.

3 Cournand, A., Riley, R. L., Breed, E. S., and Baldwin, E. de F., Interim Report, O.S.R.D. Contract OEMcmr 107, 1943.

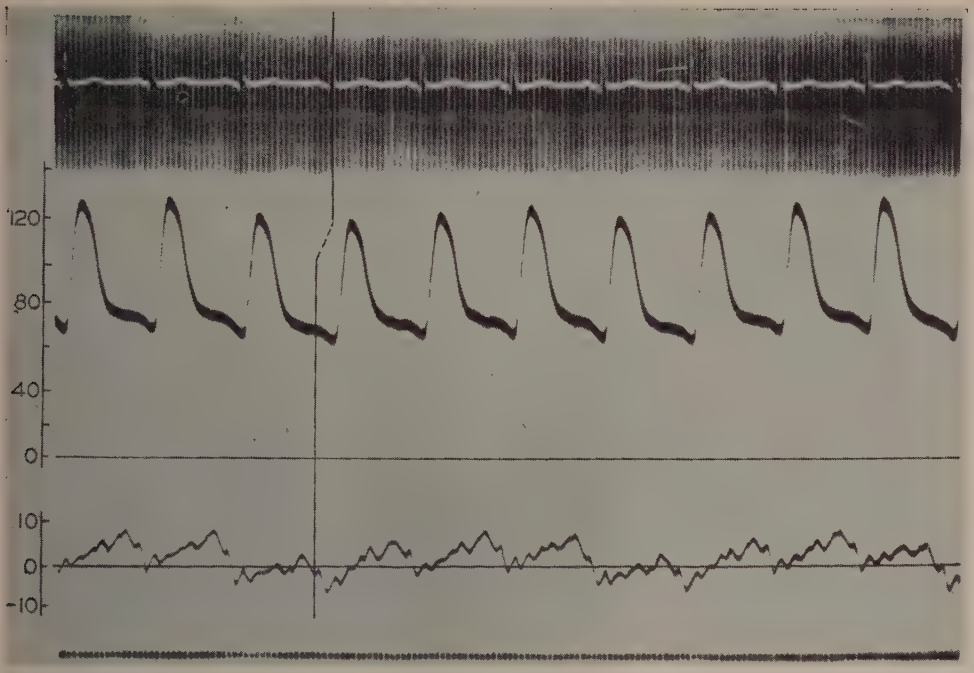

FIG. 2.
Example of simultaneous registration of electrocardiogram (upper) and pressure pulses in the femoral artery (middle) and right auricle (lower) in a patient recovering from shock. Large time divisions represent 0.2 sec. Vertical line in third cycle indicates parallax correction for the electrocardiogram. Pressures in mm mercury.

except in the presence of tachycardia or very vigorous cardiac contractions. Oscillatory phenomena were common, especially in low pressure ventricular complexes, but at present it is not certain whether some of these are artifacts dependent upon the presence of the catheter (see Fig. 1A and 2). The general contours of the pulse waves are believed to be reasonably accurate.

Results. In 8 normal subjects the right ventricular pressure averaged 22 mm mercury at the height of systole (range 18-28) and about zero during most of diastole. There was a cyclic variation of about one to 3 mm mercury due to quiet respiration (Fig. 1A). The systolic contour was full, resembling curves obtained in dogs by Wiggers.[4] In the few cases of mild and moderate essential hypertension, the pressures were not outside the normal range.

Right ventricular systolic pressures as high as 80 mm of mercury have been recorded in a case of chronic cor pulmonale. In several cases of pulmonary fibrosis or emphysema with no definite evidence of cardiac enlargement, the systolic pressure was above normal (Fig. 1B) whereas in other similar cases it was not elevated. In Fig. 1 are records of right ventricular pressure pulses from a patient with mitral stenosis and insufficiency and aortic insufficiency during decompensation (C) and after bed rest and digitalization (D).

Fig. 2 is a record of simultaneous electrocardiogram (lead II) and femoral artery and right auricular pressures from a patient recovering from shock.

Discussion. Recording pressures in the right ventricle affords a means of studying some aspects of the pulmonary circulatory

[4] Wiggers, C. J., *Pressure Pulses in the Cardiovascular System,* Longmans, Green & Co., New York and London, 1928.

dynamics in man, about which little is known. The method is being included in an investigation of the effects of pulmonary and valvular heart disease upon the cardiac output, right heart pressures, arterial pressure and peripheral resistance. Similar measurements are being made in cases of peripheral circulatory failure. The technic is also being applied to study of the cardiac cycle and its pathological alterations in man and to investigation of the pharmacological actions of some cardiovascular drugs.

14447

Effect of Alanine on Response of *Lactobacillus casei* to Pyridoxine and Folic Acid.

ESMOND E. SNELL. (Introduced by L. R. Hac.)

From the University of Texas, Biochemical Institute, and the Clayton Foundation for Research, Austin.

Previous work[1] has shown that in the presence of sufficient quantities of dl-alanine, *Streptococcus lactis* R did not require pyridoxine or "pseudopyridoxine"[2,3] while in the absence of these quantities of alanine, pyridoxine or compounds with pyridoxine activity were required for growth of this organism. From structural considerations, it appeared possible that alanine might serve as a precursor for the active substance derived from pyridoxine.[1] It was further found that *Lactobacillus casei*, an organism which also requires pyridoxine[4,5] (or pseudopyridoxine) for growth, could not dispense with this substance when excess alanine was present in the medium. It was observed, however, that when limited amounts of pyridoxine were present, growth of *L. casei* was considerably heavier when alanine was also added than it was in the absence of alanine. This effect of alanine has been investigated in connection with other growth-factor requirements of *L. casei*, with results presented below.

Experimental. The medium and technic used are the same as those previously described in detail[2] except that the medium was modified by the addition of 1 mg of asparagine, 3 γ of pyridoxine hydrochloride, and 1 γ of p-aminobenzoic acid per 10 cc. With these additions, this medium becomes, except for minor variations, almost identical with that proposed by Landy and Dicken[6] for estimation of 6 B vitamins by use of *L. casei*. Various vitamins were omitted in turn from this medium, and the quantitative growth response to additions of the vitamin determined in the presence and absence of dl-alanine. This was employed at a level of 2 mg per 10 cc. Results are given in Table I. Without exception, addition of dl-alanine improved response to the vitamin in the concentration range used for assay. This improvement, scarcely detectable with riboflavin, becomes slightly more marked with pantothenic acid and nicotinic acid. With pyridoxine and folic acid, the difference becomes so great as to alter considerably the nature of the dose-response curves, especially in that portion which would be used for assay. These differences, obtained turbidimetrically after 24 hours incubation, are also evident when acidimetric measurements are made after 72 hours incubation (Table II). In the pyridoxine

1 Snell, E. E., and Guirard, B. M., *Proc. Nat. Acad. Sci.*, 1943, **29**, 66.

2 Snell, E. E., Guirard, B. M., and Williams, R. J., *J. Biol. Chem.*, 1942, **143**, 519.

3 Snell, E. E., PROC. SOC. EXP. BIOL. AND MED., 1942, **51**, 356.

4 Snell, E. E., and Peterson, W. H., *J. Bact.*, 1940, **39**, 273.

5 Bohonos, N., Hutchings, B. L., and Peterson, W. H., *J. Bact.*, 1942, **44**, 479.

6 Landy, M., and Dicken, D. M., *Lab. Clin. Med.*, 1942, **27**, 1086.

TABLE I.
Effect of dl-Alanine on Response of *Lactobacillus casei* to Various Vitamins.

Vitamin added	Amt. per 10 cc γ	Galvanometer reading* No alanine added	Galvanometer reading* dl-Alanine added
Riboflavin	.0	3.0	4.0
	.02	11.0	12.5
	.04	18.0	19.0
	.07	27.5	28.0
	.10	42.0	40.0
	.20	63.0	62.0
	.40	73.0	68.0
	1.0	76.0	72.0
Calcium pantothenate	.0	2.0	3.0
	.02	7.7	6.8
	.04	17.5	16.2
	.07	28.0	29.0
	.10	39.3	45.0
	.15	51.7	54.5
	.30	63.0	69.0
	1.0	69.0	76.0
Nicotinic acid	.0	11.0	13.0
	.02	20.0	21.0
	.04	26.3	28.0
	.07	35.0	38.0
	.10	44.0	47.0
	.20	60.0	66.0
	.40	69.0	70.0
Pyridoxine hydrochloride	.0	7.5	18.0
	.1	14.5	27.5
	.2	18.5	35.0
	.4	25.0	42.0
	.7	31.5	47.0
	1.0	38.5	49.0
	2.0	53.0	54.0
	3.0	62.0	55.0
	mg units		
Folic acid†	.0	8.0	9.0
	.03	16.5	21.5
	.07	24.0	34.0
	.10	29.0	39.0
	.15	36.5	47.0
	.20	41.0	51.0
	.30	55.0	55.0
	1.0	73.0	62.0

* Distilled water reads zero; a reading of 100 represents opacity. Cultures were read after 24 hours incubation at 37°C.
† "Potency" 3100.[10]

test, there is displacement of turbidity and acid production upward, but the response to added pyridoxine is not greatly sensitized. In the folic acid test, there is no upward displacement of the blank, but the response to a given amount of folic acid is considerably increased, so that the organism in effect becomes more sensitive to folic acid. In neither case is the maximum growth obtained increased by the presence of dl-alanine.

In view of the increased sensitivity of *L. casei* to folic acid evident in the presence of additional dl-alanine, an experiment was designed to determine whether the response of *S. lactis* R to folic acid[7] was similarly affected (Table III). No significant difference was produced with this organism by added alanine, either in the above medium, or in the medium used by Mitchell and Snell[7] for folic acid assay. As shown previously[1] no response to pyridoxine can be obtained with *S. lactis* R in the presence of these quantities of alanine, since alanine alone produces maximum growth.

TABLE II.
Effect of dl-Alanine on Response of *L. casei* to Pyridoxine and Folic Acid as Determined Acidimetrically.

Substance added	Amt per 10 cc γ	cc N/10 acid produced* No alanine added	cc N/10 acid produced* dl-Alanine added
Pyridoxine hydrochloride	.0	.84	3.29
	.1	1.23	3.30
	.2	1.71	3.36
	.4	2.42	3.96
	.7	3.63	4.84
	1.0	4.73	5.95
	2.0	8.42	8.70
	3.0	10.0	9.37
	mg units		
Folic acid†	.0	1.08	0.97
	.03	1.68	2.89
	.07	2.51	4.40
	.10	3.63	6.38
	.15	4.75	7.90
	.20	6.93	8.80
	.30	8.80	10.10
	1.0	10.30	10.1

* 3 days' incubation at 37°.
† Potency 3100.

TABLE III.
Effect of dl-Alanine on Response of *S. lactis* R to Folic Acid.

Mg units* per 10 cc	Galvanometer reading† No alanine added	Galvanometer reading† dl-Alanine added
0	7.0	7.0
.02	26.0	27.0
.04	36.5	37.0
.07	46.0	47.0
.10	52.0	52.0

* Potency 3100.
† Incubated 16 hours.

[7] Mitchell, H. K., and Snell, E. E., *Univ. of Texas Pub.*, 1941, **4137**, 36.

TABLE IV.
Turbidimetric Assays for Folic Acid with *S. lactis* and *L. casei* in Presence and Absence of Added Alanine.

Concentrate	Relative potency* for *S. lactis*		Relative potency for *L. casei*	
	No alanine added	dl-Alanine added	No alanine added	dl-Alanine added
I	3,860	3,890	3,230	2,900
II	5,420	5,400	3,630	3,660
III	37,400	37,300	20,400	26,500
IV	79,200	79,300	42,300	66,600

* Potencies in all cases were compared to that of an empirical standard of Liver Fraction B, assigned potency = 1.0.[10] *S. lactis* was incubated 16 hours, *L. casei* for 24 hours before culture turbidities were determined.

Various workers[8,9] have reported differences in the relative activities of concentrates or pure compounds in promoting growth of *S. lactis* and *L. casei* on folic acid-free media. The relative potencies of a number of concentrates derived from spinach by methods previously described[10] were determined with both organisms in the presence and absence of dl-alanine, to see if differences in sensitivity of *L. casei* to folic acid under these conditions would significantly alter the results. The data obtained are given in Table IV. With *S. lactis,* the potencies obtained in the presence and absence of alanine are identical within experimental error. The relative potencies of these same concentrates for *L. casei* is generally lower than for *S. lactis*. In 2 of 4 cases the apparent potency is significantly higher in the presence of alanine than in its absence; but the magnitude of change is not sufficient to explain the discrepancy in assay results obtained with the two organisms.

Discussion. The favorable effect of alanine on response of *L. casei* to various vitamins on the above medium is not readily explained. According to the data of Hutchings and Peterson,[11] presence of alanine in the basal medium is not required for growth of this organism; hence it probably is able to synthesize alanine. Furthermore, the 50 mg of hydrolyzed casein present in each tube of medium supplies about 0.9 mg of alanine, if present figures for the alanine content of casein are correct. All of the effects described in the present paper can be produced by addition of one milligram of alanine per tube. Similarly, on this medium, pyridoxine or compounds derived from pyridoxine are required for growth of *S. lactis;* an additional milligram of alanine eliminates this requirement.[1] Obviously, these effects of alanine are dependent upon a comparatively high concentration of it; and the concentration supplied with the hydrolyzed casein of the basal medium is insufficient to produce these effects.

Only in the pyridoxine test is growth in the blank tubes increased significantly by added alanine. This suggests that cells of the inoculum contain a substance or substances in the presence of which alanine can be utilized in place of pyridoxine by *L. casei,* as it is by *S. lactis*.[1]

The fact that the effect of alanine on growth is more pronounced in the case of the folic acid and pyridoxine assays than in tests for the other vitamins and is scarcely discernible in at least one assay (riboflavin), suggests a somewhat specific influence on metabolism of these substances, rather than an entirely general effect in increasing vigor of the test organism.

Summary. Addition of dl-alanine to a basal medium similar to that recommended for assay of 6 B vitamins with *Lactobacillus casei*[6] somewhat improves response to the vitamins when they are present in concentra-

8 Stokstad, E. L. R., *J. Biol. Chem.*, 1943, **149**, 573.

9 Keresteszy, J. C., Rickes, E. L., and Stokes, J. L., *Science,* 1943, **97**, 465.

10 Mitchell, H. K., Snell, E. E., and Williams, R. J., *J. Am. Chem. Soc.*, 1941, **63**, 2284; *J. Am. Chem. Soc.*, in press.

11 Hutchings, B. L., and Peterson, W. H., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 36.

tions limiting growth. The effect varies in magnitude with different vitamins, and is especially pronounced with pyridoxine and with folic acid. The relative effectiveness of concentrates of the latter in promoting growth of *L. casei* may differ considerably when determined on 2 media which differ only in their alanine content.

14448

Effect of Sulfonamides on Coenzyme I-Linked Enzyme Systems.*

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At present the best theory for the mode of action of the sulfonamides postulates their inhibition of certain metabolic reactions due to the similarity in structure of the sulfonamides and the prosthetic groups of the respiratory enzymes. Evidence in support of this theory has been contributed by studies on the inhibition of the respiration and growth of bacteria, and also by the discovery that p-aminobenzoic acid has a counteracting effect on sulfonamides. Particular emphasis has been placed on the reactions involving coenzyme I, because its active group, nicotinamide, is chemically similar to the pyridine ring of sulfapyridine and to the isosteric ring of sulfathiazole. This work has been summarized by Dorfman and Koser[1] in a paper on the inhibitory effect of sulfapyridine and sulfathiazole on the nicotinamide stimulated respiration of the *dysentery bacillus*. Since all of the previous work has dealt with respiration in the whole cells of bacteria, an attempt was made to reproduce this effect in isolated enzyme systems.

For this purpose, reactions were employed in which coenzyme I was necessary for maximum activity, and where a quantitative relationship could possibly be traced between the amount of coenzyme I needed for stimulation, and the amount of sulfa drug needed for inhibition. The first reaction tried was that of the yeast fermentation method for the quantitative determination of coenzyme I.[2,3,4] The method used was essentially that of Axelrod and Elvehjem,[4] except that it was found necessary to add 0.1 mg muscle adenylic acid and 20 μg cocarboxylase to each respirometer flask for maximum carbon dioxide evolution. Coenzyme I was used at levels of 5 and 10 μg per flask.

Sulfapyridine or sulfathiazole in solution as its sodium salt (10 mg per cc) was added to the flasks with the rest of the constituents before the addition of the yeast preparation. Amounts added varied from 1-10 mg per flask.

With the final pH of the solution in the flask adjusted to 6.2-6.6 so that the normal fermentation of the yeast was not inhibited by the alkaline reaction of the sodium sulfonamides, no inhibition occurred even at the highest level of the drug. The use of the hexose diphosphate at twice the normal level, with the coenzyme I at 5 μg or 10 μg per flask did not alter the results.

Another approach to the problem was the use of enzymes present in normal rat liver which require coenzyme I in their hydrogen-transport systems. One such system—malic

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We are indebted to Merck and Company, Inc., Rahway, New Jersey, for supplies of cozymase.

† Wisconsin Alumni Research Foundation Fellow.

1 Dorfman, A., and Koser, S. A., *J. Infect. Dis.*, 1942, **71**, 241.

2 von Euler, H., *Ergebn. Physiol.*, 1936, **38**, 1.

3 Myrback, K., in Nord, F. F., and Weidenhagen, R., *Ergebnisse der Enzymforschung*, 1933, **2**, 139.

4 Axelrod, A. E., and Elvehjem, C. A., *J. Biol. Chem.*, 1939, **131**, 77.

oxidase—has been studied by Potter.[5] The livers were obtained from healthy stock rats weighing from 200-250 g. The reaction was followed by measurement of the oxygen uptake in a Warburg respirometer at 37°. Each flask contained: M/4 phosphate buffer (pH 7.6), 0.4 cc; M/2 malate, 0.2 cc; M/2 glutamate, 0.2 cc; cytochrome *c*, 4×10^{-4} M, 0.1 cc; coenzyme I, 5 mg per cc (50% pure), 0.3 cc; M/10 nicotinamide, 0.3 cc; 2% liver homogenate[6] in water, 1.0 cc; and water to make 3 cc. 0.3 cc 10% NaOH was placed in the center well of each flask to absorb the carbon dioxide evolved. Glutamate was added to aminate the oxalacetate formed, which would otherwise have inhibited the reaction; nicotinamide was added to help prevent the immediate destruction of coenzyme I. The sodium salt of sulfapyridine or sulfathiazole was added to the flasks in amounts varying from 3-10 mg. Succinylsulfathiazole was also tested at 0.4 mg per flask.

In no case did the Q_{O_2} vary significantly from the control value.

Obviously, experiments could not be limited to systems containing coenzyme I as a hydrogen carrier, since the sulfonamides might also inhibit other metabolic reactions. Hence, the effect of sulfapyridine, sulfathiazole, and also succinylsulfathiazole was tried in the succinoxidase system.[7,8] A procedure similar to that employed for malic oxidase was followed. Each flask contained the following: M/4 phosphate buffer (pH 7.4), 0.4 cc; M/2 succinate, 0.3 cc; cytochrome *c*, 4×10^{-4} M, 0.1 cc; Ca^{++} and Al^{+++} as chlorides, 0.012 M, 0.1 cc each; 0.5 cc 2% liver homogenate in water; and water to make 3 cc. The sodium salt of sulfapyridine or sulfathiazole was added in amounts varying from 1-5 mg; succinylsulfathiazole was added to the limit of its solubility, 0.5 mg per flask.

It was evident from the data obtained that the results of these experiments were also completely negative and that none of these compounds affected the activity of succinoxidase.

Sulfapyridine, sulfathiazole, and succinylsulfathiazole also had no effect on the respiration of rat liver in the presence of pyruvate or fumarate in simple systems containing only homogenate, substrate and buffer, but with coenzyme I and nicotinamide added to the fumarate system.

An attempt was also made to determine if administration of large doses of a sulfonamide to a living animal would have any effect upon the respiration of its tissues. When doses of 0.25-0.40 g of sodium sulfapyridine were given orally or intraperitoneally to normal, healthy stock animals (6 rats) 3-6 hours before death, or daily for several days, the activity of the systems was not altered from the normal values. The ranges of Q_{O_2} values, as determined under our conditions, on many normal animals, are as follows: succinate, 80-100; malate, 60-75; pyruvate, 8-14; fumarate, 9-16.

Discussion. It is unlikely that p-aminobenzoic acid had any effect in the present studies. The tissue samples would contain about 0.1 μg of p-aminobenzoic acid and the other constituents of the reaction mixtures would contribute only minute amounts. One part of p-aminobenzoic acid counteracts less than 1000 parts of sulfapyridine or sulfathiazole in *in vitro* bacterial growth studies. Since 1000 to 5000 μg of the sulfa drug were added in our experiments, the ratio of drug to p-aminobenzoic acid was probably too high for counteraction to take place. Furthermore, sulfapyridine or sulfathiazole presumably may interfere with nicotinamide coenzymes in such a manner that p-aminobenzoic acid is not involved. Dorfman and Koser[1] have found that p-aminobenzoic acid has no effect on sulfapyridine inhibition of resting cell respiration of *dysentery bacilli* but that nicotinamide or coenzyme I can reverse this effect.

The results obtained in the present studies add to the increasing evidence that sulfapyridine or sulfathiazole interfere with the production and/or utilization of nicotinamide rather than with the function of the pyridine

[5] Potter, V. R., *Advances in Enzymology*, Vol. 4, in press.

[6] Potter, V. R., and Elvehjem, C. A., *J. Biol. Chem.*, 1936, **114**, 495.

[7] Potter, V. R., *J. Biol. Chem.*, 1941, **141**, 775.

[8] Potter, V. R., and Schneider, W. C., *J. Biol. Chem.*, 1943, **149**, 217.

nucleotides themselves. McIlwain[9] has reported that pyridine 3-sulfonic acid inhibits the conversion of nicotinic acid to nicotinamide by *Staph. aureus*. Teply, Axelrod, and Elvehjem[10] found that nicotinamide, nicotinamide-ribose nucleoside and coenzyme I were of about equal potency in partially counteracting sulfapyridine bacteriostasis of *L. arabinosus*. Considerably larger amounts of nicotinic acid had no effect. The fact that both Teply *et al.*[10] and Dorfman and Koser[1] found larger amounts of coenzyme I to be necessary in the presence of sulfapyridine than under normal conditions does not necessarily invalidate the theory that the drug does not interfere with the function of the coenzyme I molecule. It is possible that coenzyme I is broken down before it is taken in by certain bacterial cells. It is also possible that in the respiration of intact cells, coenzyme I is continually broken down and sulfapyridine or sulfathiazole interferes with its resynthesis. This latter viewpoint is considerably strengthened by the work of Morel[11] who found that coenzyme I disappears from Proteus cells metabolizing pyruvate, and its content can be maintained if nicotinamide is supplied. She concludes that the coenzymes "wear out" while functioning only if there is an occasional irreversible change of the nicotinamide.

Summary. Our results indicate that sulfonamides do not interfere directly with the functioning of coenzyme I in yeast fermentation or in several systems in normal rat liver. The available experimental evidence does suggest the possibility that the drugs may inhibit the synthesis of the coenzymes.

9 McIlwain, H., *Brit. J. Exp. Path.*, 1940, **21**, 136.

10 Teply, L. J., Axelrod, A. E., and Elvehjem, C. A., *J. Pharm. and Exp. Therap.*, 1943, **77**, 207.

11 Morel, M., *Ann. inst. Pasteur*, 1941, **67**, 285.

14449

Structural Particularities of Antifibromatogenic Steroids: Experiments with Δ^4-androstene-3,17-dione and Cholestenone.*†

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The senior writer has called attention to the fact that the naturally occurring steroids which prevent abdominal fibroids induced by estrogens in the female guinea pig were all Δ^4-3-keto-steroids,[1] as progesterone, desoxycorticosterone, dehydrocorticosterone, and testosterone. There was apparently a parallelism between the progestational and antifibromatogenic activities of these substances; the antifibromatogenic activity of progesterone was greatly superior to that of testosterone propionate[7] or of Δ^{16}-dehydroprogesterone which was not antifibromatogenic.[3] The question arises how other Δ^4-3-keto-steroids known to have little or no progestational activity might behave when administered simultaneously with fibromatogenic quantities of estrogens. Two naturally not occurring

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† This work has been done in collaboration with Dr. F. Giral, of the Instituto Politécnico and of "Laboratorios Hormona" in México City, who suggested the experiments with cholestenone. Thanks are due to Dr. F. Giral for this steroid and to Dr. Karl Miescher of Ciba Pharmaceutical Products in Basel for androstenedione.

1 Lipschütz, A., *Rev. Med. y Alim* (Chile), 1941, **4**, 329; *Revue Canad. de Biol.*, 1943, **2**, 92.

2 Lipschütz, A., Vera, O., and González, S., *Cancer Research*, 1942, **2**, 204.

3 Lipschütz, A., Bruzzone, S., and Fuenzalida, F., *Rev. Med. y Alim.* (Chile), in press.

TABLE I.

Comparative Fibrous Tumoral Reaction in Castrated Female Guinea Pigs with Subcutaneous Tablets of Alpha-estradiol and Different Δ^4-3-keto-steroids. Necropsy 90 to 95 Days after Implantation.

Estradiol absorbed per day, μg	Δ^4-3-keto steroid absorbed per day, μg	Avg fibrous tumoral effect F.T.E.*	No. of animals, total	No. of animals reaching avg F.T.E. of estradiol group	Avg No. of tumoral values 2 and 3 per animal	Uterine wt, g
16-57	0	5.7	23	12	1.9	4.9 (2.7-12.0)
	Androstenedione					
25-71	157-567‡	*4.6*	10	3	1.4	3.3 (2.0-5.7)
	Cholestenone					
12-17	60-158	*5.2*	6	3	1.8	3.8 (2.0-5.3)
	Progesterone					
21-63	13-24‡	1.4	14	0	0	3.0 (1.7-5.1)
	Testosterone†					
18-49	43-59‡	1.4	9	0	0.1	2.8 (1.0-5.5)

* The "fibrous tumoral effect" or F.T.E. represents the sum of 4 numerical values relating to the size of tumors of the uterus (subserous, mesometrial), "apical" tumors (or tumors of the mesosalpinx), tumors of the digestive tract and the abdominal wall, and splenic tumors. The tumors of each of these 4 regions are arranged in 4 classes characterized by the values 0.5, 1, 2, and 3, according to the size given in Fig. 1 in the paper of Lipschütz *et al.*[7]

† The *free* steroid has been used in these experiments. Fibroids occasionally occurred even when 200 μg of testosterone propionate per day were absorbed. See the diagram on page 86 in Lipschütz, A., *Cold Spring Harbor Symposia on Quant. Biol.*, 1942, **10**, 79.

‡ Non-selective absorption from tablets of the Δ^4-3-keto-steroid mixed with cholesterol.

Δ^4-3-keto-steroids were tried: androstenedione and cholestenone. According to older statements[4] Δ^4-androstenedione is even less progestational in the rabbit than free testosterone; according to new work the progestational activity of these two androgens coincides.[5] Cholestenone is seemingly void of any hormonal action in the body.[6]

Experiments were done in the manner customary in this Department: tablets of the steroids mentioned were implanted into castrated female guinea pigs simultaneously with estrogenic tablets which when alone present in the body induce, in the course of 3 months, abdominal fibroids in most animals, with an average fibrous tumoral effect of about 5 according to the classification explained previously.[7] Androstenedione (M.P. = 172°)‡ was mixed with 20% of cholesterol to give hard tablets; one to 2 tablets of 25 to 30 mg each were implanted subcutaneously. Absorption of androstenedione was not selective.‡ Cholestenone (M.P. = 78-80°) was used without any admixture; 3 to 4 tablets were implanted simultaneously. Necropsy was made 90 to 95 days after implantation of tablets. The results are given in Table I.

All animals receiving androstenedione had abdominal fibroids. The average F.T.E. was 4.6. It is remarkable that there were large abdominal fibroids even in those cases in which 400 μg of androstenedione per day or more were absorbed. The significance of this finding becomes evident when one remembers that about 13 to 24 μg of progesterone per day are sufficient to produce a very striking antifibromatogenic action (see Table I). Comparison with testosterone also is of interest. Though fibroids induced by estradiol

[4] Klein, M., and Parkes, A. S., *Proc. Roy. Soc., B*, 1937, **121**, 574.

[5] Selye, H., and Masson, G., *J. Pharm. and Exp. Therap.*, 1943, **77**, 301.

[6] Selye, H., *Endocrinology*, 1942, **30**, 437.

[7] Lipschütz, A., Bellolio, P., Chaume, J., and Vargas, L., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 164.

‡ Determinations of M.P. and of the percentage of cholesterol at the end of the experiments were made by Dr. F. Fuenzalida of this Department.

may occasionally occur even when 200 μg of testosterone per day are absorbed[2] they do not usually occur with an absorption of only 40 μg per day (see Table I). The difference in results with Δ^4-androstenedione and testosterone were not due to differences in their masculinizing faculty. Though the capon unit of androstenedione is about 8 times that of testosterone[8] the quantities of androstenedione used in the present work were so considerable that the typical penis-like organ[9] was present in all animals. The corpus cavernosum reached in one case (with 500 μg per day) a length of 5 mm and the horny styles a length of 2 mm.

Sixty to 158 μg per day of cholestenone (M.P. = 78-80°) were absorbed. This is 10 times more than the antifibromatogenic threshold of progesterone. Abdominal fibroids were present in all 6 animals, and the average F.T.E. was not less than with estradiol alone.

As judged by the average F.T.E. androstenedione and cholestenone were not antifibromatogenic. Other criteria also supported this conclusion: the percentage of animals reaching the average F.T.E. of the estradiol group was slightly if at all influenced by androstenedione or cholestenone. The same is true concerning the average number of tumoral marks of classes 2 and 3 (tumors with a diameter of not less than 3 millimeters).

Uterine weight was smaller than with estradiol alone. Because of great variations we cannot say whether the difference is significant, but this may be an indication that with quantities still greater some antiestrogenic action might be obtained with the two substances.

Summary. Quantities of Δ^4-androstene-3-17-dione up to 40 times greater than the antifibromatogenic threshold of progesterone were not sufficient to prevent abdominal fibroids induced by alpha-estradiol. These quantities of androstenedione were also many times greater than the antifibromatogenic quantities of testosterone. The masculinizing action on the clitoris of the guinea pig was so pronounced as with testosterone. Quantities of cholestenone 10 times those of progesterone revealed no antifibromatogenic activity.

8 Goldberg, M. W., *Ergebn. d. Vitamin u. Hormonforsch.*, 1938, **1**, 37, 403, 405.

9 Lipschütz, A., *The Internal Secretions of the Sex Glands*, Cambridge (England), Baltimore, 1924, 301.

14450

Differential Behaviour of the Liver Towards Natural and Artificial Estrogens.*

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It has been known since the work of Biskind and associates that estrogens absorbed from intrasplenic pellets are inactivated in the liver.[1] The same has been stated concerning intrasplenic injections of estrogens.[2,3] These statements have been applied also to the study of abdominal fibroids induced by

* This work has been aided by the Jane Coffin Childs Memorial Fund for Medical Research, and the Rockefeller Foundation. Thanks are due to Prof. C. E. Dodds for hexestrol; to Messrs. Squibb and Sons, New York, and Instituto Sanitas, Santiago, for stilbestrol; to Dr. Karl Miescher in Basel and Dr. E. Oppenheimer of Messrs. Ciba Pharmaceutical Products in Summit for natural estrogens.

1 Biskind, G. R., and Mark, J., *Bull. Johns Hopkins Hosp.*, 1939, 212. Quoted from Biskind, G. R., and Meyer, M. A., *Endocrinol.*, 1941, **28**, 217.

2 Segaloff, A., and Nelson, W. O., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 33.

3 Fels, E., *Medicina* (Buenos Aires), 1942, **2**.

estrogens in the female guinea pig. Quantities of estradiol or its esters which induce abdominal fibroids when absorbed from subcutaneously implanted tablets or pellets do not induce fibroids when absorbed from the spleen.[4] When the same quantities are absorbed from pellets implanted into the liver, large abdominal fibroids may be produced also, but their incidence diminishes greatly.[5] The difference between results with intrasplenically and intrahepatically implanted pellets has been tentatively explained by the assumption that with intrahepatic pellets the whole quantity absorbed was put into contact with but a small fraction of the liver and that the inactivating faculty of the liver was a *limited* one. It was thought worth while to study the problem of a quantitative limitation of the inactivating faculty of the liver by using different *artificial* estrogens, as diethylstilbestrol and hexestrol. These artificial estrogens are known to be relatively more potent by oral application than the natural ones; up to 20% or more of the quantity administered by injection may appear in the urine as against 2 to 3% with alpha-estradiol.[6] Following intravenous injection diethylstilbestrol behaves differently from estrone and alpha-estradiol also concerning excretion in bile.[7] When given to female guinea pigs by subcutaneous injection in oil the artificial estrogens were found to be more likely to produce abdominal fibroids than free alpha-estradiol or estrone.[8]

Experiments. Pellets of stilbestrol or of hexestrol[9] were implanted subcutaneously,

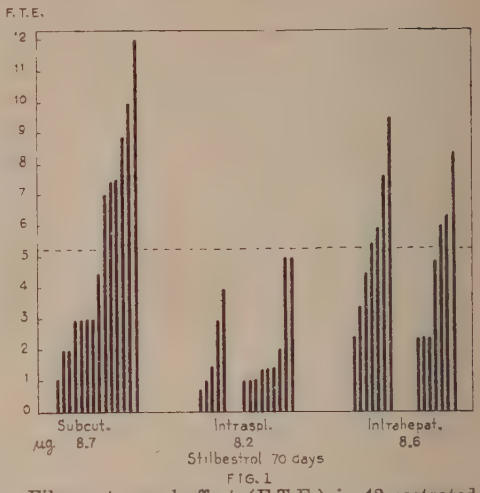

FIG. 1

Fibrous tumoral effect (F.T.E.) in 42 castrated female guinea pigs with subcutaneous, intrasplenic, or intrahepatic pellets of diethylstilbestrol, without adhesions (first group of columns) or with adhesions (second group of columns). Duration of the experiment—70 days. F.T.E. in units as explained in the foregoing paper. Horizontal line—average of the subcutaneous group. Note the enormous difference between the subcutaneous and intrasplenic groups; small or no difference between the subcutaneous and intrahepatic groups.

intrasplenically, or intrahepatically into 152 castrated female guinea pigs.† Necropsy was performed 70 to 100 days later according to the group; the fibrous tumoral reaction was classified in units as in our former work[10] (see also the foregoing paper). The results with diethylstilbestrol in an experimental series of 70 days duration are given in Fig. 1. In a number of cases adhesions between the spleen and the abdominal wall were found. These adhesions consisted sometimes of thin and unconspicuous strands between the spleen and the wall, or between the epiploon and pancreas and the wall ("without adhesions," in the diagram), the epiploon being normally in contact with the under pole of the spleen. In other cases the strands between the spleen

4 Acuña, L., Tesis Universidad de Chile, 1942, *Public. Med. Exp. Chile,* No. 12; Lipschütz, A., and Acuña, L., *Rev. Med. Alim.* (Chile), 1943, **5**, 310.

5 Carrasco, R., Tesis Universidad de Chile, 1943, *Public. Med. Exp., Chile,* No. 18; Lipschütz, A., and Carrasco, R., *Rev. Med. y Alim.* (Chile), to be published.

6 Stroud, S. W., *J. Endocrinol.,* 1939, **1**, 201.

7 Cantarow, A., Rakoff, A. E., Paschkis, K. E., Hansen, L. P., and Walking, A. A., *Endocrinology,* 1942, **31**, 515.

8 Lipschütz, A., Vargas, L., Egaña, E., and Bruzzone, S., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 467.

9 Campbell, N. R., Dodds, E. C., and Lawson, W., *Nature,* 1938, **142**, 1121.

† The greater part of these experiments will be described in U. Quintana, Tesis Universidad de Chile, 1943 (*Public. Med. Exp.* (Chile), No. 22).

10 Lipschütz, A., Bellolio, P., Chaume, J., and Vargas, L., PROC. SOC. EXP. BIOL. AND MED., 1941, **46**, 164.

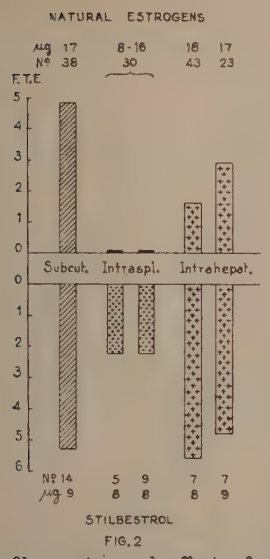

FIG. 2

Average fibrous tumoral effect of 42 animals with pellets of stilbestrol and 143 animals with pellets of natural estrogens as estrone, estradiol, and different esters.[4,5] Duration—70 days. The first intrasplenic and intrahepatic columns—without adhesions; the second intrasplenic and intrahepatic columns—with adhesions. See also the number of animals (No.) in each group and the average quantities absorbed per day. The greater resistance of the artificial estrogens against hepatic inactivation is evident.

and the abdominal wall were more extensive; in rare cases the spleen may adhere directly to the abdominal wall ("with adhesions" in the diagram). As seen in Fig. 1, there was some fibrous reaction also with intrasplenic pellets of stilbestrol. This was remarkable since a fibrous reaction was never observed with intrasplenically implanted alpha-estradiol and its esters.[4] The fibrous reaction was no less pronounced with intrahepatic pellets of stilbestrol than with subcutaneous pellets (Fig. 1) where as with intrahepatic pellets of natural estrogens the incidence of large fibroids was greatly diminished. Comparative results with natural and artificial estrogens are given in Fig. 2. Results with 19 animals with pellets of hexestrol with an absorption of 5 to 6 µg per day were similar to those with stilbestrol. These experiments afford evidence of the greater resistance of artificial estrogens to inactivation in the liver.

The evidence of greater resistance of artificial estrogens to inactivation in the liver was corroborated by the fact that other toxic actions of prolonged treatment with estrogens were present with intrasplenic pellets of stilbestrol and hexestrol such as genital bleeding, monstrous uterine growth, uterine polyps, great development of the nipples and of the mammary glands. Most of these toxic actions were absent with intrasplenic pellets of natural estrogens or they were present only exceptionally and transiently.

Summary. Experiments with intrasplenic and intrahepatic pellets of diethylstilbestrol and of hexestrol showed that these artificial estrogens are more resistant against hepatic inactivation than the natural ones. Abdominal fibroids could not be induced with intrasplenic pellets of natural estrogens, free or esterified; they were induced with intrasplenic pellets of diethylstilbestrol and of hexestrol though the incidence of tumors of a certain size was greatly diminished when compared with subcutaneously implanted pellets. Other toxic actions characteristic of prolonged treatment with estrogens also were present with intrasplenic pellets of artificial estrogens, whereas they were mostly absent or only transient with intrasplenic pellets of natural estrogens.

14451

Duration of Sleep Produced by Pentobarbital Sodium in Normal and Castrate Female Rats.*

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Cameron[1] reported an "unexpected result" —that following a given dose of pentobarbital sodium (nembutal), castrate female rats slept twice as long as did normal female rats. Specifically, he found the mean duration of sleep to be 223 ± 12 minutes for the castrated animals, and 100 ± 4 minutes for the normal rats following 40 mg of pentobarbital per kilo of body weight. He attributed the longer sleep of the castrated rats to the established tendency of castration to depress metabolism.[2,3] The depression in metabolism, he thought, would decrease detoxification and increase the susceptibility to barbiturates. Barbiturates in certain doses are known to depress oxygen consumption and body temperature.[4,5,6] Consequently in the presence of a depressed metabolic state, it is logical to assume that the duration of the action would be lengthened.

The difference of average duration of sleep between normal and castrate rats was so striking that we believed the experiment worthy of repetition. To our surprise we found that the normal animals slept about twice as long as did the castrate rats (Table I); and, in fact, our data are numerically quite similar to those of Cameron but the effect is reversed. Thus the normal rats slept 261 ± 69 minutes and the castrate rats slept 118 ± 59 minutes (Test 2) following the subcutaneous administration of 30 mg† of pentobarbital sodium per kilo of body weight.

The data in Table I are in line with the findings of Holck *et al.*[7] who have shown that spayed female rats develop a tolerance to pentobarbital more readily than do normal female rats. Holck *et al.* used another method for observing the effect of castration and his rats had been spayed for a somewhat shorter period. They observed that larger doses of pentobarbital are required to produce a given effect in spayed female rats; our data show a greater effect (duration of sleep) in normal female rats following a stated dose.

We are at a loss to explain the contradiction between our data and those of Cameron. However, on repeated tests at room temperature (Table I-B), at lower temperatures (Table I-A), and at higher temperatures (Table I-C), the normal rats uniformly slept significantly longer than did the castrate rats in each case. We are inclined to attribute the shorter duration of sleep of the castrate rats to their extra subcutaneous fat. Cameron showed that warmth tended to decrease the duration of sleep following pentobarbital The insulating effect of the subcutaneous fat would tend to maintain a higher body temperature in castrate rats. It may be seen from Table I that castrate rats averaged about 290 g body weight while the normal rats averaged about 240 g; these were significant differences in every case. The castrate rats had been operated at the age of one month and were not

* This work was supported in part by a grant from the Carnegie Corporation of New York.

1 Cameron, G. R., *Proc. Roy. Soc. Med.*, 1938, **32**, 309.

2 Loewy, A., and Richter, P. F., *Arch. Anal. Physiol.*, 1899, **174**, supp. 175.

3 Loewy, A., *Zentralblatt Physiol.*, 1902, **16**, 449.

4 Anderson, H. H., Chen, M. Y., and Leake, C. P., *J. Pharm. Exp. Therap.*, 1930, **40**, 215.

5 Dameshek, W., Myerson, A., and Loman, J., *Am. J. Psychiat.*, 1934, **91**, 113.

6 Shapiro, L. B., *J. Nerv. Ment. Dis.*, 1937, **85**, 305.

† We found that 40 mg per kilo body weight—the dose used by Cameron—resulted in unfavorably high mortalities.

7 Holck, H. G. O., Mathieson, D. R., Smith, E. L., and Fink, L. D., *J. Am. Pharm. Assn.*, 1942, **31**, 116.

TABLE I.
Data on Duration of Sleep of Rats Given 30 mg of Pentobarbital per kg Body Weight.

Test No.		No. rats	Avg body wt, g	Avg induction time, min.	Avg time asleep, min.	No. asleep	Probability of significant difference: N-C (P)	No. rats died
			Part A: Air Temperature—13° C.					
4	Normal	18	243	31	247 ± 105	16		1
							.15-.10	
	Castrate	17	292	36	168 ± 113	10		1
6	Normal	17	248	8	292 ± 118	16		0
							<.01	
	Castrate	17	295	29	161 ± 95	13		0
			Part B: Air Temperature—23°C.					
2	Normal	20	243	23	261 ± 69	20		1
							<.01	
	Castrate	19	297	45	118 ± 59	16		1
7	Normal	16	244	36	276 ± 90	16		1
							<.01	
	Castrate	17	293	62	166 ± 70	12		0
			Part C: Air Temperature—37°C.					
3	Normal	17	243	31	173 ± 42	16		1
							<.01	
	Castrate	18	296	39	109 ± 84	12		1
5	Normal	19	236*		186 ± 78	19		0
							.02-.01	
	Castrate	19	287		88 ± 52	15		0

* Avg wt of 17 rats.

tested experimentally until 12 months later. This is the procedure‡ that Cameron had followed, and he reported that his castrate rats weighed 240 to 355 g whereas his normal rats weighed from 160 to 350 g.

The average induction time was greater in each instance for the castrate rats than for the normal rats (Table I). The induction time was taken as the interval between the time of injection subcutaneously of the pentobarbital solution along the lateral portion of the abdominal skin and the time when the rats could be lifted carefully from the cage and laid, still asleep, on the cage top. The duration of sleep was taken as the interval between the time of this transfer and the time when the rat had sufficiently awakened to move itself off the cage top. Both the induction time and the "time asleep" were quite variable. The induction time for the normal rats averaged about 30 minutes in most tests, but was as high as 45 to 62 minutes (Tests 2 and 7) for the castrate rats. Only rarely did all of the rats go to sleep (Table I); the average "time asleep" is that of the rats which slept. In each test the normal rats' duration of sleep was significantly greater than that of the castrate rats; the probabilities calculated according to Fisher's "t" test[8] are given in Table I.

The mortality did not differ significantly between the normal and castrate groups, 4 of the normal rats died during the various experiments and 3 of the castrates died. This also is not in accord with Cameron's finding that 22% of the castrate rats died and none of the normal rats. However Holck *et al.*[7] have shown that 2 to 3 times as much pentobarbital is required to kill the average spayed female rat as to kill the normal female control

‡ Cameron used rats aged 12 months which had been spayed at age 1 month. Thus, Cameron's rats were 1 month younger than ours; this difference in age is probably negligible.

[8] Fisher, R. A., *Statistical Methods for Research Workers*, London, 7th edition, 1938, 128.

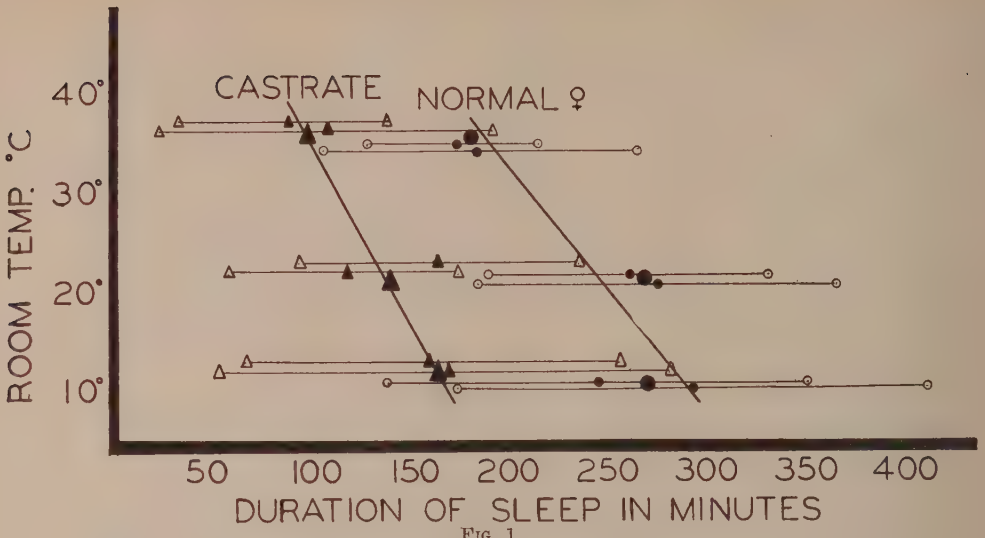

FIG. 1.

Duration of sleep at various room temperatures. Although wide variations in the average durations of sleep in both normal and castrate female rats are observed at each room temperature, there are consistent tendencies toward shorter sleep periods with increases in room temperature.

rat. Our results are in accord with Holck's; castration increases the resistance of the female rat to pentobarbital.

Both the normal and castrate rats showed a tendency to a shorter duration of sleep at increasing temperatures (Fig. 1). The differences between the durations of sleep at the lower temperature (13°C) and at room temperature (23°C) were of doubtful significance for both the normal and castrate groups. However, significant differences were obtained in each case between the durations of sleep at room temperature and at the higher environmental temperature (37°C). This finding is in agreement with the observations of Cameron and also with the clinical observation that barbiturate poisoning may be treated by fever.

Summary. Normal and castrate female rats were given 30 mg of pentobarbital per kilo of body weight subcutaneously. The normal rats slept significantly longer than did the castrate rats. Both groups of rats were exposed to various environmental temperatures, specifically 13°, 23°, and 37°C. In general, the duration of sleep decreased with the increase of environmental temperature.

14452

Effect of Testosterone Propionate and Methyl Testosterone on Creatinuria in Progressive Muscular Dystrophy.

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Progressive muscular dystrophy is a disease in which large groups of voluntary muscles undergo primary degeneration and atrophy. The disease is hereditary and familial, although isolated cases are encountered. Atrophy is marked in all forms, but hypertrophy or pseudohypertrophy is an early and prominent symptom in one form. Numerous attempts have been made to understand the nature of the process underlying this disorder, to the end that effective treatment may be found. Failure thus far to evolve a basis for therapy has characterized most of the results of studies on this disease, although enthusiastic claims have been advanced from time to time only to have been discarded at the hands of sober and objective therapeutists.

During a period of 2 years, in which we have had 40 cases of progressive muscular dystrophy under study, we have been impressed, as have other workers, with the marked predilection of this disease for males, in whom the disease, if established early, tends to run a much more acute course than in the occasional female in whom the disease is encountered. This observation, together with the fact that the disease in the male tends to become less acute following the onset of puberty, leads one to the hypothesis that the essential aberration in progressive muscular dystrophy is one connected closely with the biological phenomenon of maleness on the one hand and, perhaps, with those systems concerned with growth and development on the other. Since certain of these phenomena are mediated through the hormones of the anterior pituitary, the adrenal cortex, and the gonads, it was believed that an investigation of the effects of these agents on the metabolism and clinical course of patients with this disease should be carried out, in so far as modern technics of endocrinology permit.

A number of reports have appeared recently concerning the effects of the steroid hormones, particularly testosterone, on the growth and development of the muscular system, and more specifically on the metabolism of creatine.[1–5] On the whole, however, reports have been equivocal, and in some instances flatly contradictory. Creatinuria has long been regarded as the most consistent abnormality in the metabolism of patients with progressive muscular dystrophy. Any agent, therefore, which affects the excretion of creatine, should be of interest in a study of this syndrome. Accordingly, a study of the effects of a number of the available members of the steroid hormone series on the metabolism of creatine, creatinine, and glycocyamine, in normal subjects and in patients with progressive muscular dystrophy, was begun. Five children between the ages of 7 and 12 years, in whom progressive muscular dystrophy was moderately advanced, were chosen for study. Since a somewhat variable physiological creatinuria is encountered in the pre-adolescent male, 4 normal children of comparable ages were selected as control subjects. Control subjects and patients with muscular dystrophy were placed on diets of constant composition and base line values were obtained for the excretion of creatine, creatinine and glycocyamine.

* Member of the Naval Unit at the Hospital of The Rockefeller Institute for Medical Research. The Bureau of Medicine and Surgery does not necessarily endorse views or opinions expressed in this paper.

1 Nitzescu, I., and Gontzea, I., *C. R. Soc. biol.*, 1937, **125**, 80.

2 Jailer, J. W., *Am. J. Physiol.*, 1940, **129**, 389; 1940, **130**, 503.

3 Coffman, J. R., and Koch, F. C., *J. Biol. Chem.*, 1940, **135**, 519.

4 Duckworth, D. A., *J. Clin. Endoc.*, 1942, **2**, 13.

5 Sutton, M. B., *J. Clin. Endoc.*, 1941, **1**, 882.

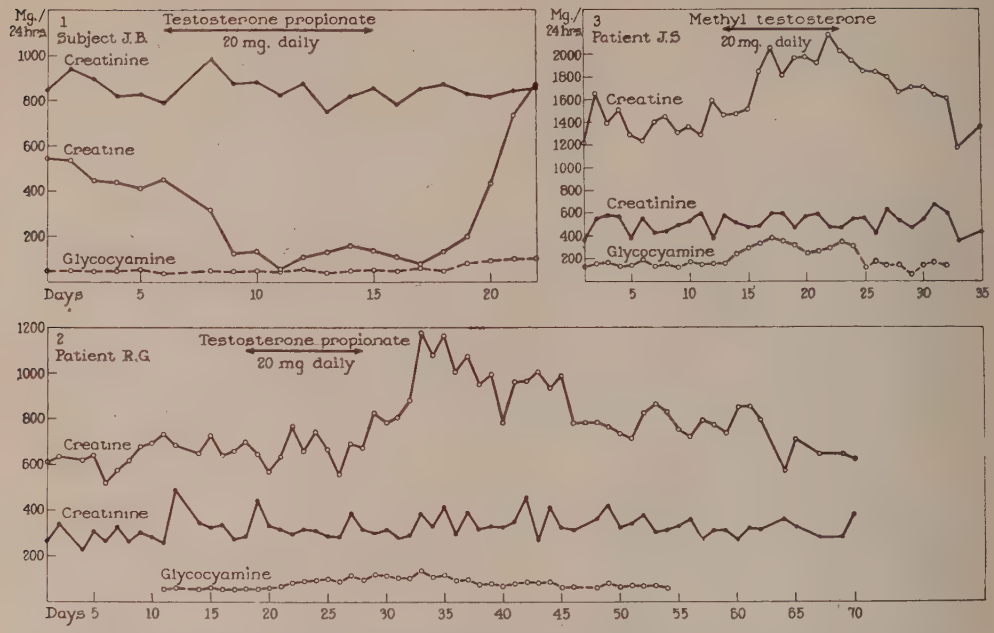

FIG. 1.

The excretion of creatine, creatinine, and glycocyamine in the urine of normal subjects and patients with progressive muscular dystrophy following the administration of testosterone propionate and methyl testosterone.

The determination of glycocyamine was included in these studies because of its known role as an intermediary substance in the biological synthesis of creatine from glycine, arginine, and methionine,[6,7] and because its excretion is known to be altered in patients with muscular dystrophy.[8] After a control period during which the excretion of these substances was found to be constant from day to day, testosterone propionate† was administered intramuscularly to normal subjects and patients, in doses of 20 mg a day, over a period of 10 to 14 days. The hormone was then discontinued and determinations of creatine, creatinine, and glycocyamine were continued until approximately the former levels of excretion of these substances were reached.

Certain differences in the effect of testosterone on the excretion of creatine and glycocyamine in normal subjects and in patients exhibiting muscular dystrophy were immediately apparent. There was a marked drop in the excretion of creatine in each of the normal controls within 72 hours after the administration of testosterone propionate which persisted as long as the hormone was continued. Immediately upon withdrawal of the hormone, however, there was a marked creatinuria which was maintained for several days. Results on a typical normal subject are given in Fig. 1 (No. 1, Subject J.B.). In the patients exhibiting dystrophy (No. 2, Patient R.G.), on the other hand, there was no significant change in the output of creatine during the interval in which the hormone was administered. However, as in the normal subjects, following withdrawal of testosterone, a marked increase in the excretion of creatine

[6] Borsook, H., and Dubnoff, J. W., *J. Biol. Chem.*, 1941, **138**, 389.

[7] DuVigneaud, V., Chandler, J. P., Cohn, M., and Brown, G. B., *J. Biol. Chem.*, 1940, **134**, 787.

[8] Gilder, H., Shank, R. E., and Hoagland, C. L., unpublished data.

† We are greatly indebted to Roche-Organon, Inc., Nutley, N.J., for the testosterone propionate (Neo-Hombreol) and methyl testosterone (Neo-Hombreol M) used in these studies.

and glycocyamine was observed, which lasted for a period of several days, after which there was a slow decrease to the former base line values. No significant change in creatinine excretion was apparent.

In contradistinction to testosterone, methyl testosterone has been shown to produce creatinuria in normal subjects.[9] However, creatine storage is said to occur with this agent,[10] and the accompanying creatinuria has been explained as being due to an accelerated synthesis of creatine.[9] Accordingly, 2 patients with progressive muscular dystrophy were given 20 mg of methyl testosterone† orally for a period of 10 days, and analysis of the urine for creatine, creatinine, and glycocyamine was performed as before. The results obtained with a typical patient are given in Fig. 1 (No. 3, Patient J.S.). In contradistinction to the effects observed with testosterone there was a marked increase in creatine excretion as soon as the administration of methyl testosterone was begun, and the increase was maintained as long as the hormone was continued. A marked increase in urinary glycocyamine likewise occurred. Upon withdrawal of the hormone, however, no further increase occurred in the output of creatine or glycocyamine. Indeed, there was a rapid drop in the excretion of both creatine and glycocyamine until the previous levels of output were approximated.

One possible explanation for the marked output of creatine occurring in patients with progressive muscular dystrophy, following the withdrawal of testosterone, is that creatine storage had occurred as a result of the administration of the hormone in spite of the fact that no marked changes in creatine excretion were demonstrated while the hormone was being administered. In other words, although creatine was retained during the period of hormone administration, the storage was not sufficient to be reflected in significant changes in a high creatine output over the relatively short periods covered by the analyses. In a later patient, in whom the disease was in its earliest stages, and the creatinuria only slightly above normal, a definite decrease in creatine output *was* observed soon after the administration of the hormone was begun.

The administration of testosterone has been claimed to produce an increase in the deposition of creatine in the muscles of normal animals, as revealed by direct measurement of the concentration of creatine in the muscle,[10] and by a marked retention of creatine as reflected in a drop in its excretion in the urine.[3,10,11] No reports have been found, however, of studies on the creatine output of patients with progressive muscular dystrophy following the giving of this hormone. If creatine retention, which occurred as a result of the testosterone medication, may be regarded as a true storage phenomenon, it would indicate that in progressive muscular dystrophy the capacity to store creatine is not irreversibly altered. Moreover, it would justify the administration of the hormone in therapeutic amounts over a prolonged interval of time in order to learn if muscle function can be improved thereby. Studies directed toward this end are underway and the results will be reported in a subsequent communication.

9 Samuels, L. T., Henschel, A. F., and Keys, A., *J. Clin. Endoc.*, 1942, **2**, 649.

10 Pizzolato, P., and Beard, H. H., *Endocrinology*, 1939, **24**, 358.

11 Williamson, M., and Gulick, A., *Endocrinology*, 1941, **28**, 654.

14453

A Color Reaction of Ascorbic Acid with Nicotinamide and Nicotinic Acid.

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Since reactions between different vitamins are always of interest, it appears worthwhile to report the following observations. In the course of performing a number of spot tests, it was observed that a yellow color was produced when a mixture of ascorbic acid and nicotinamide was moistened with water and stirred into a thick paste. The formation of the canary yellow color was almost immediate. Nicotinic acid gave a similar color reaction with ascorbic acid although the reaction proceeded more slowly. None of the other vitamins that were tested gave this reaction; these included pyridoxine hydrochloride, thiamine, calcium pantothenate, and para-amino benzoic acid.

Failure to note this reaction previously in any of the numerous mixtures of vitamins used as dietary supplements probably was due to the common practice of including riboflavin in such preparations. The deep yellow color of the riboflavin would mask that of the reaction.

Whether the reaction alters the biologic activity of the vitamins or represents one of the functions of ascorbic acid in the body is not known.

14454

A Color Reaction of Ascorbic Acid with Derivatives of Pyridine, Piperidine, Quinoline, and Iso-Quinoline.

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Recently it was shown by T. H. Milhorat[1] that ascorbic acid will react with nicotinamide and nicotinic acid with the production of a yellow color. A number of other vitamins tested gave no color reaction with the ascorbic acid. The present investigations show that many pyridine derivatives, other than nicotinamide and nicotinic acid, show this color formation with ascorbic acid, and for the numerous compounds tested so far the reaction has been specific for certain definite chemical structures.

While the color formation usually can be observed when ascorbic acid is mixed with these substances in solution, the simplest and most satisfactory method is to mix a small amount of each on a white porcelain spot plate and stir into a paste after the addition of a drop or two of water. In most instances, the color formation is almost immediate; in the case of a few substances a short period of from 10 to 20 seconds might be required for the full development of the color.

Observations. A large number of substances representing various types of chemical structure were tested. The chemical compounds which gave a yellow color reaction with ascorbic acid are shown in Fig. 1. The list of substances giving no color reaction is too long to reproduce here but those most important for the purposes of studying the specifici-

1 Milhorat, T. H., PROC. SOC. EXP. BIOL. AND MED., 1944, **55**, 52.

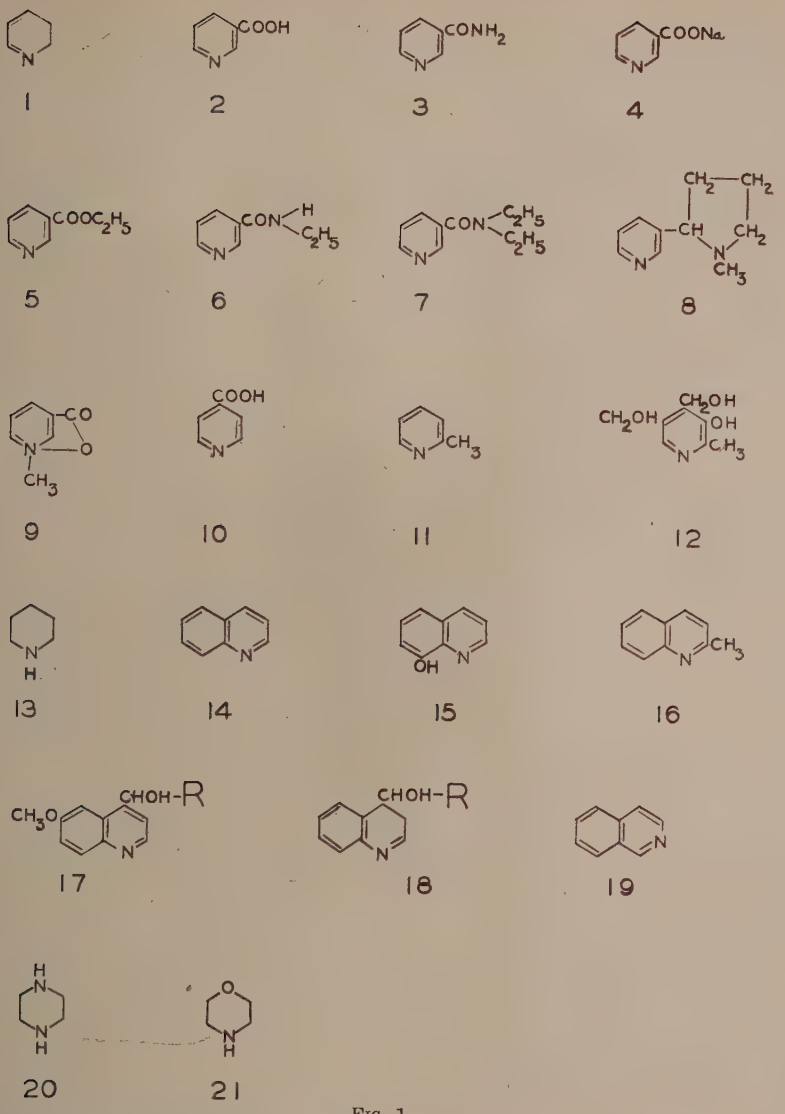

FIG. 1.
Substances Giving a Yellow Color Reaction with Ascorbic Acid.
1. Pyridine; 2. nicotinic acid; 3. nicotinic acid amide; 4. sodium nicotinate; 5. ethyl nicotinate; 6. nicotinyl monoethylamide; 7. nicotinyl diethylamide; 8. nicotine; 9. trigonelline; 10. isonicotinic acid; 11. alpha-picoline; 12. pyridoxine (vitamin B_6); 13. piperidine; 14. quinoline; 15. 8-hydroxyquinoline; 16. quinaldine; 17 quinine and quinidine and their various salts (hydrochloride, dihydrochloride, sulphate, hydrosulphate, ethyl carbonate); 18. cinchonine and cinchonidine; 19. isoquinoline; 20. piperazine; 21. morpholine.

ty of the reaction are illustrated in Fig. 2. Other compounds giving no reaction included tryptophane, histidine, cystine, cysteine, methionine, leucine, isoleucine, valine, norvaline, proline, aminoacetic acid, aspartic acid, threonine, lysine, alanine, phenylalanine, ornithine, arginine, various guanidine derivatives, ephedrine and several chemically related compounds, adenine, picrotoxin, strychnine, brucine, veratrine, physostigmine, sparteine, digi-

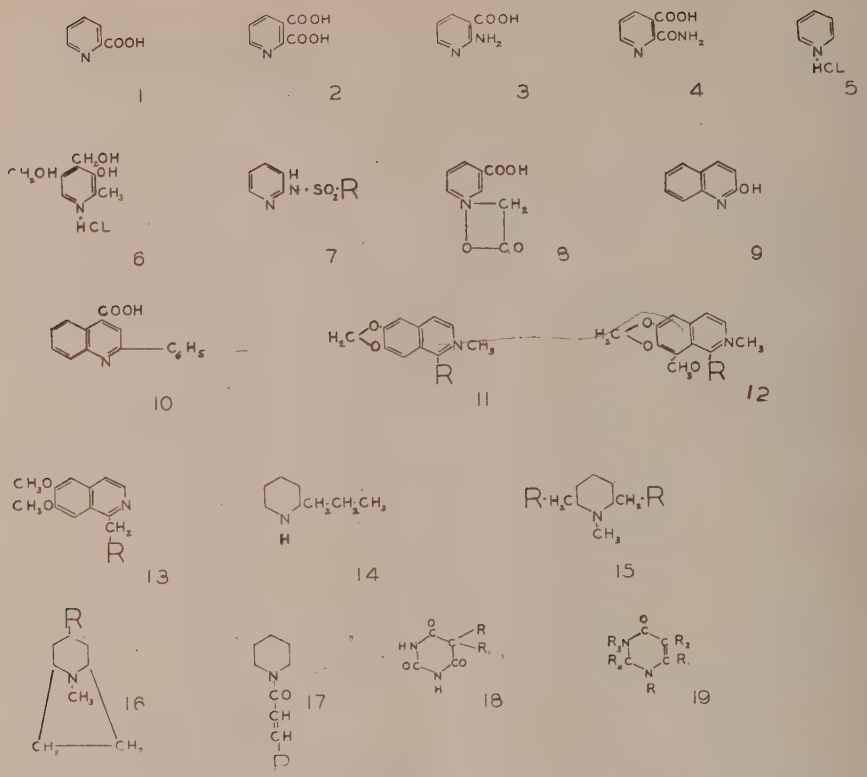

FIG. 2.
Some of the Substances Giving No Color Reaction with Ascorbic Acid.
1. Picolinic acid; 2. quinolinic acid; 3. 2-amino nicotinic acid; 4. quinolinamic acid; 5. pyridine hydrochloride; 6. pyridoxine hydrochloride; 7. sulfapyridine; 8. pyridine-betaine-3-carboxylic acid; 9. 2-hydroxy-quinoline; 10. cinchophen; 11. hydrastine; 12. narcotine; 13. papaverine; 14. coniine; 15. lobeline; 16. atropine and hyoscyamine; 17. piperine; 18. phenobarbital and other derivatives of barbituric acid; 19, caffeine, theobromine, guanine, uric acid.

talin, phenol and various derivatives, benzoic acid, hydroquinone, menthol, camphor, isoborneol, anthracene, thiamine, pantothenic acid, vitamin K, and several sterols.

In general, the observations indicate that the color reaction occurs when the alpha-carbon of the pyridine ring is not occupied by a radical other than the methyl group. These conditions hold also with the piperidines, quinolines, and iso-quinolines that were investigated. The radicals interfering with the reaction are shown in Fig. 2.

The two betaines, trigonelline and pyridine-betaine-3-carboxylic acid are of interest in that an intense yellow color was produced when they were treated with strong alkali alone. This reaction was not observed with any of the other derivatives of nicotinic acid. No color formation was observed when ascorbic acid was mixed with piperine which has a substitution on the nitrogen atom; however, treatment of piperine with acid alone produced a brilliant yellow color. Ascorbic acid gave a yellow color with trigonelline, but not with pyridine-betaine-3-carboxylic acid. Failure of this latter substance to give the color reaction with ascorbic acid might be due to steric hindrance or the presence of a strongly acidic radical attached to the nitrogen atom. It has also been shown that the hydrochlorides of pyridine and pyridoxine do not react with ascorbic acid, whereas the free bases do.

Picolinic acid, quinolinic acid, and cinchophen, substances in which an acid radical is

attached to the alpha-carbon, did not react with color formation with ascorbic acid unless the acid radical was neutralized. Of the many substances investigated, morpholine and piperazine were the only compounds which gave the color reaction and were not derivatives of pyridine. However, both compounds may be considered as piperidine in which the CH_2 group in the gamma position has been replaced by either O or NH. In both instances the development of color was slow in contrast with the almost immediate formation of color in the case of nicotinamide and other derivatives of pyridine.

Other sugars and related substances substituted for ascorbic acid gave no color reaction with nicotinamide; those tested included glucose, levulose, xylose, mannose, lactose, galactose, sorbitol, arabinose, inositol, glycogen, and gluconic acid.

The reducing property of the ascorbic acid, as determined by titration with dichlor-phenol-indo-phenol, was found to be unchanged by the color reaction with nicotinamide. Furthermore, the effect of an ingested dose of 25 mg ascorbic acid on the urinary output of the vitamin in a fasting dog was essentially the same whether or not the ascorbic acid had reacted with nicotinamide before its administration to the animal. Whether the biologic activity of nicotinamide is affected by the reaction is not known.

Summary. Ascorbic acid reacts with formation of yellow color with nicotinamide, nicotinic acid, and a number of other derivatives of pyridine. In general, a radical other than the methyl group attached to the alpha-carbon atom interferes with the reaction. Similar conditions were observed in the case of the piperidines, quinolines, and iso-quinolines that were investigated. The effect on the reaction of radicals attached to the nitrogen atom and the influence of neutralization of a carboxyl group in the alpha position are discussed.

14455

Vitamin E and Length of Life of Rats Fed a Diet with Fatally Low Protein Content.*

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The experiments reported here were originally planned as part of an investigation designed to determine whether a condition simulating exudative diathesis could be induced in rats fed with a vitamin E-deficient diet. The exudative diathesis occurs in chicks when the diet is deficient in vitamin E and contains highly unsaturated fatty acids, such as those provided by cod liver oil, and, within certain limits, can be accelerated by a high carbohydrate-low protein ratio in the diet.[1] Since it is also known from clinical medicine that a tendency to edema is enhanced when the diet is high in carbohydrate and low in protein, it was thought that a drastic change of the vitamin E-deficient diet in this same direction might precipitate similar symptoms in rats. Although no condition resembling exudative diathesis occurred in these animals it was observed that adequacy of vitamin E enabled the rats to survive longer on the protein-insufficient diet than was the case when the diet was also lacking in vitamin E. Although further study of this problem is not immediately contemplated it seems worthwhile reporting the experimental data since the results may offer some additional suggestions concerning the physiological functions of vitamin E.

Procedure. Groups of 6 newly weaned rats equally distributed according to weight in the

* Aided by a grant from the Josiah Macy, Jr., Foundation.

1 Dam, H., *J. Nutr.*, in press.

TABLE I.
Data for Groups of 6 Rats Receiving Protein-deficient Diets with or without Vitamin E.

Time, weaning until start of low protein diet, days		Wt at weaning, g		Wt start of low protein diet, g		Wt at death, g		Length of life in days on low protein diet	
		non-E	E	non-E	E	non-E	E	non-E	E groups
7		38	39	57	60	37	36	29	45
7		38	33	58	61	33	35	70	45
7		37	40	55	61	35	34	59	43
7		40	40	61	62	48	38	30	43
7		35	33	58	53	44	34	24	43
7		38	39	59	54	37	39	19	43
	Avg	37.7	37.3	58.0	58.5	38.8	36.0	38.5	43.7
14		40	43	75	73	51	41	45	80
14		44	44	84	69	50	35	56	55
14		44	44	83	75	56	37	49	90
14		45	37	74	58	50	30	35	75
14		43	44	78	68	50	40	43	65
14		36	40	67	71	38	40	57	52
	Avg	42.0	42.0	76.8	69.0	49.2	37.2	47.5	69.5
21		41	39	98	85	71	51	45	74
21		40	38	84	100	65	65	34	66
21		38	38	88	92	68	54	33	55
21		38	40	84	80	64	52	32	74
21		37	40	102	99	74	51	45	75
21		42	41	91	85	59	44	45	63
	Avg	39.4	39.4	91.1	90.3	67.0	52.8	39.0	67.8
42		39	41	140	168	76	82	92	116
42		41	41	151	152	112	72	54	110
42		41	40	165	150	100	72	83	84
42		40	40	144	160	68	77	108	86
42		41	39	148	138	80	88	76	84
42		41	42	168	158	124	70	37	82
	Avg	40.5	40.5	152.5	154.5	92.3	76.9	75	93.6

non-E and E groups were given the following diet, with or without the addition of 10 mg % d,l-alpha-tocopherol acetate:† alcohol-extracted casein 20 g, ether-extracted yeast‡ 10 g, sucrose 63 g, McCollum's salt mixture No. 185 7 g, diacetate of 2-methyl-1,4-naphthohydroquinone 1 mg, cod liver oil 5 g. After a given number of days the casein was removed from the diet and replaced by a corresponding amount of sucrose. The animals were kept on this diet until they died.

The results, presented in Table I, demonstrate that the animals in all groups receiving the tocopherol supplement survived somewhat longer, on the average, than those deprived of vitamin E and reached a lower weight before they died. The difference was least pronounced, and perhaps not significant, in the groups which were shifted to the low-protein diet after only one week. It may be noted that the amount of vitamin E given in the E-sufficient diets is moderately high.

No satisfactory explanation can be offered for the difference in length of life depending upon the presence or absence of vitamin E in the diet. It is possible that the deficiency of two factors in the diet, protein and vitamin E, will reduce the resistance of the animals faster than deficiency of only one factor, the protein. The effect may thus not be specific for vitamin E. It is also possible that in the presence of vitamin E the low protein intake is somewhat better utilized, although there is no direct experimental evidence for such a postulation.

† From Hoffmann-La Roche, Inc., Nutley, N.J.
‡ Prepared from Fleischmann Type 2019.

14456

Galactose-Poisoning in Chicks.*

HENRIK DAM.

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During a study concerning the significance of carbohydrate sources in the production of symptoms of vitamin E deficiency young chicks were given a diet containing 54.6% of galactose. A few days after being transferred to such a diet the animals became severely ill, sat shivering or shaking and were seized by tetanic or by clonic spasms of leg and wing muscles, often accompanied by screaming. Although the initial attacks were usually followed by temporary recovery, several additional attacks during the next few days resulted in death. The symptoms were relatively infrequent when the animals were deprived of food for some time, but occurred frequently when feeding was resumed. The body weight declined during the galactose feeding. The brain of the affected animals showed no macroscopic changes. This disorder is unrelated to an insufficiency of vitamin E in the diet since it develops much earlier than the symptoms of this deficiency and can be prevented by replacing the galactose with any other carbohydrate. The following preliminary investigation of this galactose-poisoning in chicks, which the author for practical reasons is unable to carry further at the present time, is reported in the hopes that it may be of some interest to investigators in this and related fields.

Procedure. The diet was the following: alcohol-extracted casein 15 g, ether-extracted yeast 10 g, McCollum's salt mixture No. 185 7.2 g, $CuSO_4 \cdot 5H_2O$ 0.01 g, $MnSO_4 \cdot 4H_2O$ 0.02 g, galactose 54.6 g, gelatine 8 g, gum arabic 5 g, choline chloride 0.1 g, l-cystine 0.1 g, diacetate of 2-methyl-1,4-naphthohydroquinone 0.1 mg, cod liver oil 5 g. Another group of chicks received the same diet with the exception that sucrose was used instead of galactose.

* Aided by a grant from the Josiah Macy, Jr., Foundation.

Glycogen in liver and muscles was determined by the method of Pflüger[2] modified in the following way: 1 g of the minced tissue was heated with 1 cc 60% KOH in a graduated centrifuge tube on a boiling water-bath 2 to 3 hours (covered to prevent drying), cooled and diluted with water to 4 cc. After centrifugation 3 cc of the supernatant liquid was taken out and added to 3 cc of 96% alcohol. After 15 minutes the precipitated glycogen was centrifuged down and washed twice with a mixture of 1 vol. 15% KOH and 2 vols. 96% alcohol. The precipitate was slowly dissolved in water and neutralized with conc. HCl. Water was added to make 10 cc. After heating on a boiling water-bath 3 hours the contents of the tube were cooled to room temperature and the volume readjusted to 10 cc. This solution which represents 0.75 g of tissue was used for the glucose determination after Benedict. The amount of solution taken out for the sugar determination varied from 0.5 to 2 cc according to the amount of glycogen in the organ. The sample was neutralized before the addition of the copper reagent.

Calcium was determined in the blood plasma of two birds after Clark and Collip's method.[3]

The results of the chick experiment are presented in Table I. In order to compare the galactose-disease in chicks with the corresponding disease in rats described by other authors,[4] 5 newly weaned rats were given the same galactose diet as the chicks. The results are presented in Table II.

Discussion. The brief experiments indicate

1 Somogyi, M., *J. Biol. Chem.*, 1928, **78**, 117.

2 Pflüger, E., *Pflügers Arch.*, 1903, **96**, 98.

3 Clark, E. P., and Collip, J. B., *J. Biol. Chem.*, 1925, **63**, 461.

4 Mitchell, H. S., PROC. SOC. EXP. BIOL. AND MED., 1935, **32**, 971.

TABLE I.
Blood and Tissue Analyses of Chicks Fed Diets Containing 54.6% Galactose and 54.6% Sucrose, Respectively (Non-fasting Values).

Chick No.	Age in days: Feeding began	Symptoms first observed	At death	Killed for tissue analysis	Total blood sugar, mg%	Blood galactose, mg%	Liver glycogen, mg%	Muscle glycogen, mg%	Plasma Ca, mg%
Galactose diet:									
2341	7	10	10						
2342	7	10		11				109	
2343	7	8		9	502	280	43		
2344	7	8		9	624	400	6		
2345	7	10		11				350	
2346	7	9		11	550	328	9		
2347	7	10		11				468	
2348	7	12	12						
2349	7	9	10						
2350	7	8	11						
2405	28	32		32					10.6
Sucrose diet:									
2353	7			9	252	0	420		
2355	7			11				420	
2356	7			11				312	
2357	7			11	200	0	401		
2359	7			9	208	0	464		
2360	7			11				447	
2402	28			32					10.6

TABLE II.
Blood Sugar and Liver Glycogen in Rats Reared from Weaning on Diet Containing 54.6% Galactose (Non-fasting Values).

Rat No.	Days exper. before cataract appeared	Blood sugar at the 34th day of exper.: Total mg%	Blood sugar at the 34th day of exper.: Galactose, mg%	Liver-glycogen at the 35th day of exper., mg%
71	18	254	60	1720
72	16	273	123	1470
73	12	230	53	2000
74	12	206	59	1680
75	16	370	73	1700

that the spasms observed in galactose-fed chicks are not related to low blood-sugar or to a deficiency of muscle-glycogen. Although the exceedingly low liver-glycogen and high blood-galactose appears to be an essential feature, there is as yet insufficient evidence to indicate how this is related to the symptomatology observed. It is possible that the high blood-galactose in some way or other causes a dysfunction of the central nervous system. Changes in the total oxalate-precipitable calcium in the plasma seem not to occur.

In rats the galactose-feeding did not result in low liver-glycogen and the blood-galactose was not as high as in the chicks. This indicates that rats are able to utilize galactose better than chicks. The rats did not show clinical symptoms resembling those of the chicks, but cataract developed as previously described by Mitchell[4] and the growth was subnormal, the average weight gain in 5 weeks being about 20 g.

The failure of cataract to appear in the chicks may be due to the acute and much more severe character of the disorder in this species resulting in early death before sufficient time had elapsed to induce the eye lesions. In another series of experiments 10% of galactose was added to the sucrose diet without giving rise to clinical symptoms or to disturbance of growth in chicks. A sys-

tematic investigation with varying amounts of galactose in the diet might throw some more light on this question.

Summary. Chicks fed a diet containing a high percentage of galactose develop violent spasms and die within few days. There is a high content of galactose in the blood during the spasms but about normal blood glucose. Muscle glycogen is about normal but liver glycogen nearly zero. The mechanism of the disorder is not yet clear.

14457

Use of a Germicidal Quaternary Ammonium Salt in Nutritional Studies.

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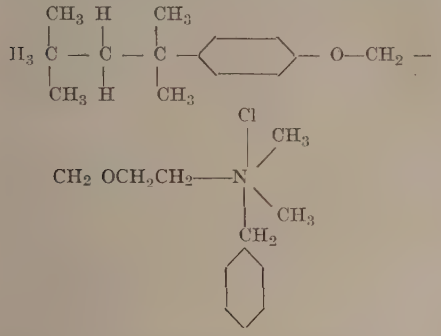
Until sterile rats can be raised routinely, studies involving synthesis of vitamins by bacteria existing in this animal will have to depend largely on the use of suitable agents to eliminate at least some of the microörganisms. Certain sulfa drugs have proven to be of much value in such studies, but it is recognized that their usefulness is limited because they attack only certain types of bacteria while others survive and indeed tend to fill in the "vacancy" created by removal of the sulfonamide-susceptible types.[1,2] It seems logical therefore that each new germicide whose properties suggest that it might be useful in nutritional studies should be given a trial. This report summarizes preliminary observations on the effect of feeding one of the quaternary ammonium salt germicides to rats on various diets.



Experimental. The compound Phemerol* was chosen because of its potency, purity, low toxicity, and availability.[3,4] In all the studies, 21-day-old, male, albino Sprague-Dawley rats were used. The basal ration consisted of sucrose 76, Labco casein 18, salts IV[5] 4, corn oil 2; vitamins (mg per 100 g ration): choline 100, nicotinic acid 0.250, calcium pantothenate 2.0, thiamine 0.200, pyridoxine 0.250, riboflavin 0.300, 2-methyl-1,4-naphthaquinone 0.100, biotin 0.010 (S.M.A. conc.) Two drops of haliver oil containing 1 mg α-tocopherol were fed weekly to each rat. When used, para-aminobenzoic acid and inositol were added at a level of 25 mg per 100 g of ration and vitamin C at 100 mg %. Three rats were used per group and growth results given in Table I are averages over a 5-week period.

It was found that when crystalline Phemerol was incorporated in the diet at a level as low as 0.1%, rats refused to eat the ration and soon died from inanition. A ration containing

1 Light, R., Cracas, L., Oliott, C., and Frey, C., *J. Nutr.*, 1942, **24**, 427.

2 Gant, O., Ransone, B., McCoy, E., and Elvehjem, C., PROC. SOC. EXP. BIOL. AND MED., 1943, **52**, 276.

* The Phemerol used in these studies was supplied by Parke, Davis and Co., Detroit, Mich.

3 Rawlins, A., Sweet, L., and Joslyn, D., *J. Am. Pharm. Assn.*, 1943, **22**, 11.

4 Joslyn, D., You, K., and Rawlins, A., *J. Am. Pharm. Assn.*, 1943, **22**, 49.

5 Hegsted, D., Mills, R., Elvehjem, C. A., and Hart, E., *J. Biol. Chem.*, 1941, **138**, 459.

TABLE I.
Growth of Rats Receiving Phemerol on Various Diets.

	Avg weekly gains in g	
	Series 1	Series 2
Basal	29	26
" + Phemerol	10 (1 dead)	11 (2 dead)
" + " + 5% Whole Liver Substance	23	26
" + " + 5% Dried Yeast	18	
" + " + 5% Grass Extr. Powder	20	
" + " + 5% Solubilized Liver Extr.	20	17
" + " + 2% " " "	15	17
" + " + 2% " " " + P.A.B. + Inositol + Vit. C		21
Basal + Phemerol + Folic Acid ≈ 2% Solubilized Liver Extr.	7 (1 dead)	
" + " + " " ≈ 4% " " " + P.A.B. + Inositol	8	

this amount of Phemerol is irritating to the throat. However, the animals readily drank a 1-1500 water solution of Phemerol as their sole source of liquid and soon became accustomed to a 1-1000 solution. Therefore, in all subsequent experiments the drug was administered in the drinking water.

Twenty-one-day-old rats usually will not survive on a 1-1000 solution of Phemerol even though they are on a diet containing 5% whole liver powder. They usually survive on a 1-1200 solution but early growth is poor. We have found it desirable to start with a 1-2000 solution and gradually work up to a 1-1200 concentration within 2 weeks. This procedure was followed in experiments reported in Table I.

Because it was feared that lipoidal material might interfere with the action of Phemerol, in the early experiments the corn oil was fed by dropper, 3 drops every third day. However, we have found that similar results are obtained if the fat is mixed in the ration and this procedure was followed in series 2.

Preliminary work involving more than a hundred rats demonstrated the following facts: (1) If a 1-1500 Phemerol solution was used as the sole source of liquid by rats on the basal diet outlined above without biotin, poor growth was obtained, but if 5% whole liver powder was added, excellent growth resulted. (2) On a synthetic ration, a 1-2000 Phemerol solution had no effect over a period of several weeks. (3) On a 1-1500 solution with no biotin in the ration, addition of 2% solubilized liver extract only partially restored the growth rate. Further addition of biotin produced some growth response and finally addition of 5% whole liver powder produced normal growth. All subsequent experiments were made with biotin in the ration.

Discussion. Results reported in Table I indicate that a combination of folic acid, para-aminobenzoic acid and inositol is not able to counteract the effects of Phemerol. Solubilized liver extract, yeast, yeast extract, and grass juice powder all partially counteract the effects of the drug at the levels fed. At equivalent levels, whole liver powder seems to be superior to solubilized liver extract, grass juice powder or dried yeast.

These results are quite different from those obtained with sulfa drugs, in which case biotin and folic acid or solubilized liver extract seem to restore the growth rate very effectively. This, of course, might be expected if Phemerol attacked different types of bacteria than did the sulfa drugs.

Since it is possible that Phemerol is inactivated by intestinal contents or is absorbed in the first portion of the G-I tract, it is interesting to consider its possible effects in the mouth, throat, and stomach. A bacterial cell which grows in the mouth, synthesizes vitamins, and is then digested and releases these vitamins would certainly be of greater nutritional significance than a large number of bacteria in the intestines which synthesized vitamins but retained them within the cells and passed out in the feces. Sherman *et al.*[6]

[6] Sherman, J., Niven, C., and Smiley, K., *J. Bact.*, 1943, **45**, 249.

have demonstrated that *Streptococcus salivarius*, which requires only 5 vitamins on a synthetic medium, occurs in large numbers in the mouths and throats of normal individuals and in some cases thrives in the intestines.

Summary. Administration of Phemerol to rats in the drinking water at a 1-1500 or 1-1200 dilution results in poor growth on a synthetic ration. Addition of 5% whole liver powder gives good growth. A combination of folic acid, para-aminobenzoic acid, and inositol does not counteract the effects of the drug.

14458

Alterations in Mammary Structure Following Adrenalectomy in the Immature Male Rat.*

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It has been reported that adrenalectomy in underfed intact and castrated female rats caused the mammary glands to grow more rapidly, to increase the number of bud-like growths along the course and at the ends of the ducts, and to increase the area covered by the gland.[1] In adrenalectomized lactating rats, the mammary alveoli were reported less distended than normally but were restored by NaCl therapy.[2] The paucity of data on the structural changes in the mammary gland subsequent to adrenal ablation suggested further investigations.

Young male rats of the Long-Evans and Sprague-Dawley strains were used, the ages ranging from 22 to 35 days at the time of adrenalectomy. An adequate stock diet plus tap water and a 1% NaCl solution was fed *ad libitum*. Bilateral adrenalectomy was performed in one operation and in experiments in which castrated males were used, the latter operation was carried out the preceding day. Autopsies were made 10-12 days later.

In some experiments, normal and castrated adrenalectomized rats, together with their controls, received 5 μg of alpha estradiol dipropionate (Di-Ovocylin)† daily for 10 days. The right abdominal mammary glands were prepared as stained whole mounts for study.

Results. The most notable change in the mammary glands which followed adrenalectomy was an increase in the number of lateral buds or short branches along the duct system. This was seen in 14 of 17 young operated males compared to 9 controls. Half of the operated rats also showed an increased number of end buds, many of which were much enlarged. The gain in body weight of the adrenalectomized males averaged 14 g less than their controls but they were otherwise in an apparently good state of health. The greatest amount of gland development occurred in those operated animals which gained the most weight. Similarly, the glands in 6 of 10 adrenalectomized castrated males also showed a greater number of lateral duct buds when compared to those from 8 castrated controls. End buds were not observed to be stimulated. The areas of gland tissue in the adrenalectomized animals in both groups were not more extensive than in the controls.

When estrogen treatment was given to 5 adrenalectomized and 4 control male rats, the increased number of lateral buds in the experimental group showed a marked dilatation. The average body weight gain in the adrenalectomized injected group was only half that of the controls. As before, the degree of lateral bud proliferation varied directly with the amount of weight gained.

Similarly, if castrated animals were used, and treated with estrogen, the mammary

* Aided by a grant from the Sackett Fund of the Trustee-Faculty Committee on Research.

1 Butcher, E. O., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 571.

2 Levinstein, Irving, *Anat. Rec.*, 1937, **67**, 477.

† Acknowledgment is made to Dr. E. Oppenheimer of Ciba Pharmaceutical Products, Inc., Summit, N.J., for a generous supply of hormone.

glands of 9 of 10 adrenalectomized rats showed more development than was found in 6 controls. In some cases, the end buds showed no enlargement, but the increased number of short lateral branches surpassed that of any mammary gland seen in these experiments.

Discussion. In some respects, these findings agree with those of Butcher[1] but differ in that they were obtained in normally fed rats. If the animals failed to gain appreciably in weight, the mammary glands did not show the specific effects of adrenalectomy. Neither did the areas of the glands from adrenalectomized rats exceed those of the controls, in contrast to the results of Butcher.[1] Estrogen stimulation superimposed on that of adrenalectomy seemed to emphasize the effect of the latter. The variation in results obtained might well be attributed to variable amounts of functioning accessory cortical tissue known to be present. In one experimental group, in which NaCl was not given, only 2 of 5 animals showed increased lateral buds and these two gained appreciably in weight. No explanation of these mammary changes can be made at this time.

Summary. Adrenalectomy in normal or castrated immature male rats resulted in an increased number of lateral buds on the duct system of the mammary tree and in some cases increased end bud growth, particularly when body growth was not markedly inhibited. Estrogen treatment in adrenalectomized rats caused dilatation of these stimulated lateral buds or else further increased their number above that of the injected unoperated controls.

14459

Effect of Adrenocorticotropic Hormone on the Insulin Content of the Rat's Pancreas.*

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In the course of investigations concerning various metabolic effects of purified anterior pituitary hormones, both lactogenic and adrenocorticotropic hormone (ACTH) were found to increase the insulin content of the rat's pancreas.[1] The lactogenic hormone used in those studies was essentially pure while the ACTH was admittedly impure, its main contaminant being the lactogenic hormone (10 to 30%). The opinion was therefore expressed that the action of the ACTH fractions on the pancreas might be due to their contamination with lactogenic hormone, rather than representing a function of the former hormone. Since that time purified ACTH has become available[2] and it has been possible to reinvestigate this problem using ACTH preparations containing less than one percent of lactogenic hormone.‡

Hypophysectomized rats received the hormone preparations for 2 weeks, starting on the day following operation. The injections were given once daily, intraperitoneally; 3 injections of one-half the usual daily dose were distributed over the last 24 hours preceding autopsy, during which time all animals

* Aided by grants from the Research Board of the University of California and the Rockefeller Foundation, New York City.

† Present address: Western Regional Research Laboratory, United States Department of Agriculture, Albany, California.

1 Fraenkel-Conrat, H., Herring, V. V., Simpson, M. E., and Evans, H. M., *Am. J. Physiol.*, 1942, **135**, 404.

2 Li, C. H., Simpson, M. E., and Evans, H. M., *Science*, 1942, **96**, 450.

‡ We wish to thank Dr. C. H. Li for kindly placing at our disposal the highly purified ACTH here employed.

TABLE I.
Effect of Pituitary Hormones on Insulin Content of the Rat's Pancreas.*

Hormone administered	Pancreatic insulin, units/100 g rat		
	Individual Expts.	Average	S_D†
Adrenocorticotropic	0.90, 0.69, 0.53, 0.75, 0.63	0.70	0.064
Lactogenic	0.90, 0.58, 0.51, 0.66, 0.86	0.70	0.096
Adrenocorticotropic plus Lactogenic	0.60, 1.00, 0.93, 0.94, 0.55	0.80	0.078
None (controls)	0.50, 0.44, 0.51, 0.53, 0.50	0.50	

* Two-month-old hypophysectomized male rats (4 to 9 per group), receiving injections for 14 days once daily from the day following operation. Daily dose per rat, given in one intraperitoneal injection: 5.0, 4.0, 3.0, 3.0, 2.5 mg ACTH and/or 2.0, 2.0, 1.5, 1.5, 1.25 mg lactogenic hormone, respectively in the five experiments listed. During a 24-hour fast preceding autopsy, one-half the daily dose was administered three times.

† S_D is the standard error of the difference between means, according to:

$$S_D = \sqrt{\frac{\Sigma x_1^2 + \Sigma x_2^2 \times (N_1 + N_2)}{(N_1 - 1) + (N_2 - 1) \times N_1 N_2}}$$

were fasted. The determination of the insulin extractable from the pooled pancreas of each group of rats was carried out as in previous reports.[1,3]

As indicated by Table I, the insulin content of the pancreas of the rats receiving ACTH (2.5 to 5 mg daily) was found consistently higher than that of the controls, the average increase being 40%. These increases were similar in magnitude to those produced by half the dose of lactogenic hormone, concurrently administered to similar rats. The increases were not as great as those previously observed after administration of a crude ACTH preparation, believed to be rich in lactogenic hormone. The possibility of a synergism between these two hormones offered itself as an explanation for this discrepancy. Groups receiving a mixture of the two hormones were therefore also included in the above experiments. No consistent synergistic or additive action was evident in these experiments, although the highest pancreatic insulin levels observed during this study occurred in 3 groups receiving the double treatment. Statistical analysis indicated that the differences between all series of rats receiving ACTH and the controls were significant. The increases in pancreatic insulin in the groups receiving lactogenic hormone alone (at half the dose) were significant.§

Summary. Treatment with purified adrenocorticotropic hormone for 14 days has been found to increase the insulin content of the pancreas of hypophysectomized rats. The previously observed action of the lactogenic hormone has been confirmed.

[3] Fraenkel-Conrat, H., Herring, V. V., Simpson, M. E., and Evans, H. M., PROC. SOC. EXP. BIOL. AND MED., 1941, **48**, 333.

§ The fact that the present series of control groups showed lower pancreatic insulin levels than had been observed in preceding years[1] may be noted, although it cannot yet be explained. It may possibly be a consequence of the use of animals which had survived several severe insulin shocks in the breeding colony. The consistency in the insulin level of the pancreas of the present control groups is remarkable.

14460

Experimental Technic for Measuring Mean Systolic Blood Pressures During Activity, Rest, and Natural Sleep.

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Blood pressures are conventionally determined in the dog either directly by a mercury or a Hamilton[1] manometer connected to a cannula or needle introduced into a large artery or indirectly by auscultation or palpation distal to a modified Riva-Rocci[2] blood pressure cuff about an extremity or a Van Leersum[3] arterial loop. The positioning or training required for these methods precludes blood pressure determinations during normal emotional and physical activity, rest, and sleep. Accordingly reported normal blood pressure values have varied significantly, A new technic for blood pressure determination that permits frequent readings during varying phases of activity or rest was therefore devised and tested. It has not to the knowledge of the authors been previously described.

Healthy dogs were anesthetized and placed on one side on an operating table with the upper leg externally rotated. The lower half of the upper side of the trunk and the groin and thigh were then shaved and prepared. Employing aseptic technic, the iliac and the upper femoral artery was exposed above and below the inguinal ligament through a 3-inch incision. A 1-inch square of sterile fine mesh gauze was next placed beneath each artery. A small rubber balloon fashioned from ½ inch of the tip of a small finger cot and attached over a short segment of 18-gauge needle to a 4-foot length of fine plastic tubing was then placed above and in direct contact with each artery. Each gauze was folded about the artery and the balloon to form a cuff and sutured snugly in place. Care was taken to avoid arterial injury or constriction. Each cuff was carefully filled and tested by the introduction of sterile water under pressure. The 2 plastic tubes were then guided subcutaneously around the flank and out through a stab wound located over the first lumbar spine. The inguinal wound was closed and dressed. The 2 tubes were led through a 30-inch length of flexible BX cable sheath the proximal end of which was securely attached to a canvas harness or corset. A sterile dressing was placed over the stab wound and the harness was adjusted to the dog's back by 6 straps. The animal was allowed to recover from the anesthetic and then placed in a prepared animal cage.

The BX cable was then attached to a pulley arrangement so that the flexibility of the cable and the excursion of the pulley allowed the dog free movement about his cage. Each of the two plastic tubes coming through the stab wound over the first lumbar spine and passing upward through the flexible cable was then connected to a manometer system. The tube from the femoral pressure cuff was attached to a capillary glass manometer and the entire system was filled with a tinted water solution. The manometer was placed 3 feet above the cuff. The hydrostatic pressure of this open system was sufficient to transmit a wide pulsating excursion from the femoral artery to the column of tinted water in the capillary tube. The plastic tube from the iliac cuff was then connected to a pressure bottle and a mercury manometer the zero point of which had been set to correspond with the level of the cuff. Care was taken to evacuate all air from the tube and the balloon in each system. The pulse rate was determined without disturbing the animal by counting the pulsations in the water manometer. (Fig. 1.)

Blood pressure readings were obtained by inflating the balloon in the iliac cuff. When

[1] Hamilton, W. F., Brewer, G., and Brotman, I., *Am. J. Physiol.*, 1934, **107**, 427.

[2] Riva-Rocci, *Un Nuovo Sfigmomanometro, Gazz. Med. di Torino*, 1896, **50**, 51.

[3] Van Leersum, *Arch. G. d. ges. Physiol.*, 1911, **142**, 377.

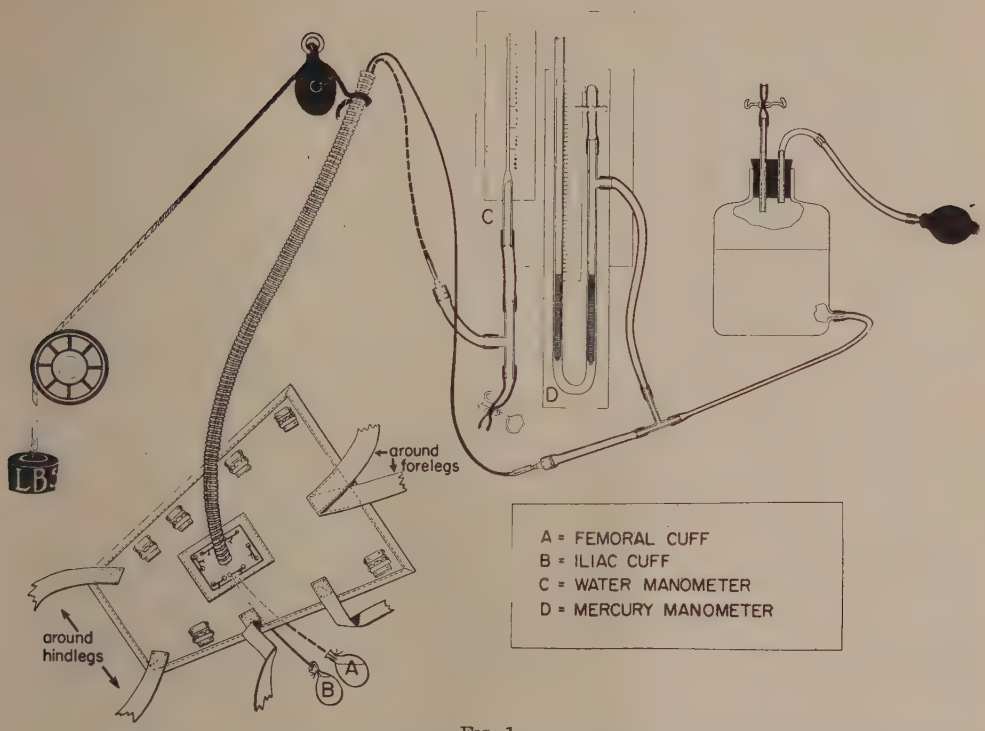

FIG. 1.

Diagram of double cuff blood pressure apparatus. Elastic sacs A and B are incorporated into cuffs about the femoral and iliac arteries. The fine plastic tubes are led subcutaneously through a stab wound to the manometers. The pulleys and flexible tubing allow free movement. Blood pressures may be recorded at will during various phases of activity and rest.

TABLE I.

Modified Double Cuff Blood Pressure Measurement in Dogs.
Each reading represents a typical determination.

	Dog 1		Dog 2		Dog 3		Dog 4	
Activity	BP	Pulse	BP	Pulse	BP	Pulse	BP	Pulse
Exercising	117	135	135	140	147	132	128	112
Standing	125	128	120	131	158	119	118	110
Voiding	—	—	119	151	150	104	—	—
Eating	129	110	126	120	161	126	100	116
Drinking	—	—	128	122	162	111	113	105
Resting	103	89	90	91	135	93	78	91
Sleeping	101	106	—	—	—	—	72	70

this pressure was sufficient to compress the iliac artery the femoral artery in the femoral cuff ceased its pulsation and decreased in size. The column in the water manometer fell and the pulse excursion disappeared. The pressure required in the iliac system to produce this phenomena was read from the mercury manometer as the mean systolic blood pressure.

This technic was employed successfully with 4 dogs and permitted repeated blood pressure determinations over periods of time varying from 3 to 6 days. Readings were taken at will without disturbing the animal or inducing symptoms of discomfort. Table I illustrates the utility of this technic by summarizing average blood pressures taken during various stages of activity, rest, and sleep.

The technic should prove useful in certain

experiments. It has the disadvantage of requiring anesthesia and surgery early and of initiating thrombosis and edema late. There is, however, a period frequently lasting several days during which the animal is bright and active and in good general condition. Observations during this period seem reasonably reliable.

Blood pressure observations employing this technic confirm the impression that there is a normal range rather than a normal blood pressure level for the dog varying with the degree and nature of activity.

14461

Streptomycin, a Substance Exhibiting Antibiotic Activity Against Gram-Positive and Gram-Negative Bacteria.*†

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With the exception of streptothricin,[1] most of the antibiotic substances known at the present time, including penicillin and other mold products as well as gramicidin and actinomycin, act largely upon gram-positive bacteria. The activity of these substances upon gram-negative organisms is highly selective, as in the case of penicillin, which affects the *Neisseria* group and has little activity upon *Escherichia coli* and other gram-negative bacteria,[2] or else much larger quantities are required to bring about the inhibition of these bacteria, as in the case of actinomycin.[3] Among the antibiotic agents that act selectively alike against both gram-positive and gram-negative bacteria, streptothricin occupies a prominent place; since this substance is water-soluble and possesses limited toxicity to animals, it is of particular interest from a chemotherapeutic point of view. Unfortunately, streptothricin has very little activity against a number of bacteria found among both the gram-negative (*Pseudomonas fluorescens, Ps. aeruginosa*) and the gram-positive (*Bacillus mycoides* and *B. cereus*) groups.

In a search for antagonistic organisms that are active against gram-negative bacteria, and from which antibiotic substances could be isolated, the actinomycetes were found[4] to offer extensive potentialities. Although most of the antibacterial agents produced by these organisms are also active against gram-positive bacteria, certain few of them exert a marked selective activity against many of the gram-negative types of bacteria. *Actinomyces lavendulæ,* which produces streptothricin, is such an organism. After detailed examination of a large number of cultures, either isolated at random from different natural and enriched soils and composts, or selected from the culture collection, another organism was found that produces an antibiotic substance which apparently combines many of the desirable antibacterial properties. This organism is similar, in most of its cultural characteristics as well as in its morphology,

* Journal Series paper, New Jersey Agricultural Experiment Station, Rutgers University, Department of Soil Microbiology.

† With partial support from a grant made by the Commonwealth Fund of New York.

1 Waksman, S. A., and Woodruff, H. B., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 207; *J. Bact.*, 1943, **46**, 299.

2 Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A., Heatley, D., Jennings, M. A., and Florey, H. W., *Lancet*, 1941, **241**, 177; *Nature*, 1942, **148**, 758; **149**, 356.

3 Waksman, S. A., and Woodruff, H. B., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 609; *J. Bact.*, 1941, **42**, 231.

4 Waksman, S. A., Horning, E. S., Welsch, M., and Woodruff, H. B., *Soil Sci.*, 1942, **54**, 281; Welsch, M., *J. Bact.*, 1942, **44**, 571.

TABLE I.
Production of Antibiotic Substances by 5 Actinomycetes.*

Organism	Nature of substance	Medium	Dilution units†			Diffusion units‡
			B. subtilis	*S. aureus*	*E. coli*	
A. antibioticus	Actinomycin	Tryptone-starch	750	500	0	—
,, ,,	,,	Nutrient broth	500	200	0	123
A. lavendulæ	Streptothricin	Tryptone-starch	500	150	75	115
,, ,,	,,	Nutrient broth	20	<30	<30	10
A. griseus (18-16)	Streptomycin	Tryptone-starch	150	30	75	123
,, ,, ,,	,,	Nutrient broth	500	30	75	190
A. griseus (D-1)	Streptomycin	Tryptone-starch	75	<30	<30	30
,, ,, ,,	,,	Nutrient broth	100	30	<30	70
Micromonospora sp.	Micromonosporin	Tryptone-starch	30	—	0	6
,, ,,	,,	Nutrient broth	100	—	0	5

* The cultures were grown in a submerged and agitated state, at 28°C for 6 days.
† Plate method used.
‡ Calculated against a streptothricin standard, using *B. subtilis* spores as the test organism.

to *A. griseus* isolated from the soil some 28 years ago.[5] The active substance is in many respects similar to streptothricin, although it differs from it in its greater activity against various gram-negative bacteria, notably the *Ps. aeruginosa* group, as well as against those aerobic spore-forming bacteria, such as *B. mycoides*, which are resistant to streptothricin. Because of its similarity to streptothricin, this substance may be designated as *streptomycin*, derived from the generic name that has recently been given to the aerial-mycelium producing and sporulating group of actinomycetes, namely *Streptomyces*.[6] Two strains of the organism producing streptomycin were obtained, one (No. 18-16) from a heavily manured field soil and the other (D-1), a somewhat less active form, from a smear plate of the throat of a chicken; it is doubtful, however, whether this organism is a normal inhabitant of the animal system.

In order to compare the antibacterial properties of the culture filtrate of the two strains of this organism with those of other actinomycetes producing antibiotic substances, the results of a typical experiment are reported in Table I. The filtrates of *A. antibioticus* and of *Micromonospora* sp. gave no activity against *E. coli;* however, *A. lavendulæ* and the 2 strains of *A. griseus* exerted an appreciable effect upon this bacterium. Results obtained by the agar diffusion method were found to be comparable to the dilution units for *B. subtilis* and *Staphylococcus aureus*. The nature of the medium in which the organisms were grown is of considerable importance in the production of the different antibiotic agents: streptothricin is produced most abundantly in a tryptone-starch medium, and streptomycin in ordinary nutrient broth, namely, a peptone-meat extract medium.

A further study of the influence of the composition of the medium upon the production of the active agent by *A. griseus* brought out the fact that, whereas streptothricin is formed abundantly in a simple medium, streptomycin requires the presence of a specific growth-promoting substance supplied by meat extract. Corn steep liquor can take the place of the meat extract. Addition of glucose further increases the yield of the substance. The nature of the protein hydrolyzate is apparently immaterial, since tryptone gave about the same degree of activity as peptone. A medium was finally adopted, consisting of 1% glucose, 0.5% peptone, 0.3% meat extract or 1.2% corn steep, and 0.5% NaCl.

The course of production of streptomycin under submerged and stationary conditions is brought out in Table II. The organism did not form any acid either in the submerged or

[5] Waksman, S. A., *Soil Sci.*, 1919, **8**, 71.

[6] Waksman, S. A., and Henrici, A. T., *J. Bact.*, 1943, **46**, 337.

TABLE II.
Metabolism of *A. griseus* and Course of Production of Streptomycin.

Shaken cultures						Stationary cultures					
	Dilution units						Dilution units				
Days	*B. subtilis*	*E. coli*	Diffusion units	pH	Growth mg	Days	*B. subtilis*	*E. coli*	Diffusion units	pH	Growth mg
2	150	40	10	7.8	270	3	40	5	6	7.7	73
3	250	50	70	7.7	185	5	150	20	12	7.8	171
4	200	40	60	7.8	—	7	225	20	53	7.9	163
7	500	125	70	8.2	—	9	275	75	—	8.3	264
						12	>300	100	55	—	—

TABLE III.
Comparative Bacteriostatic Spectra of Streptomycin and Streptothricin.
On basis of crude, ash-free dry material.

		Units of activity per gram ash-free dry material	
Organism	Gram stain	Streptomycin × 1000	Streptothricin* × 1000
B. subtilis 0	+	125	500
B. mycoides 0	+	250	<3
B. mycoides 317-911	+	20	<3
B. cereus	+	30	<3
B. mesentericus	+	15	—
B. megatherium	+	100	150
S. aureus	+	15	200
S. lutea	+	100	150
M. phlei	+	100	50
M. tuberculosis	+	30	—
Phytomonas pruni	—	100	400
Listerella monocytogenes	—	10	—
Shigella gallinarum	—	—	150
E. coli	—	25	100
S. marcescens	—	25	5
A. aerogenes	—	10	50
P. vulgaris	—	10	50
S. aertrycke	—	2.5	—
S. schottmülleri	—	—	50
Ps. fluorescens	—	2	<3
Ps. aeruginosa	—	1	<3
Cl. butylicum	—	3	<3

* These results are partly based on data reported previously; data obtained more recently with purified preparations give the same type of spectrum. Since streptothricin represented a more purified and, therefore, more concentrated preparation than streptomycin, a better comparison would be with the activity against *E. coli* as a unit; the units for the other test organisms would, therefore, have to be multiplied by 4.

in stationary cultures, as was found to be the case of *A. lavendulæ*,[7] the reaction of the medium becoming alkaline even in the presence of glucose. Growth was much more rapid in shaken cultures, although very good activity was also obtained in a stationary condition.

The growth of the organism was allowed to proceed for 5 to 12 days and the streptomycin isolated from the culture filtrate, using a method similar to that developed previously for the isolation of streptothricin.[1]

The antibacterial behavior of streptomycin, as compared with that of streptothricin, can best be illustrated by an examination of the respective bacteriostatic spectra for the two substances, as presented in Table III. Concentrated preparations, although not of the same degree of purification, of the materials were used. Taking the activity against *E. coli* as a standard, streptomycin was found to have

[7] Woodruff, H. B., and Foster, J. W., *Arch. Biochem.*, **2**, 301.

the same activity as streptothricin against *B. subtilis, A. aerogenes,* and *P. vulgaris;* it was less active against *S. aureus* and certain strains of *Salmonella;* it was much more active against *B. mycoides, B. cereus, Mycobacterium phlei, Serratia marcescens, Ps. aeruginosa, Ps. fluorescens,* and *Cl. butylicum.*

Streptomycin, like streptothricin, possesses strong bactericidal properties, and preliminary experiments tended to indicate that the two substances are also comparable in their low toxicity to animals and in their *in vivo* activity. The various chemical and biological properties of streptomycin tend to point to this compound as one closely related to streptothricin; the fact that it differs from the latter in the nature of its antibacterial activity may indicate a closely related but not the same type of molecule.

Summary. A new antibacterial substance, designated as streptomycin, was isolated from two strains of an actinomyces related to an organism described as *Actinomyces griseus.* This substance resembles streptothricin in its solubility in water, mode of isolation and concentration from culture medium, its selective activity against gram-negative bacteria, and its limited toxicity to animals. However, the two substances differ in the nature of their respective bacteriostatic spectra as well as in their quantitative action upon different bacteria. It is suggested that one is dealing here with two closely related chemical compounds.

14462

Production of Riboflavin Deficiency in the Monkey.*

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In a recent report from this laboratory[1] it was demonstrated that the rhesus monkey (*Macaca mulatta*) grows well and remains in excellent health for at least 14 months on a highly purified diet consisting of sucrose 73, purified casein† 18, salts 4, corn oil 3, cod liver oil 2, and adequate quantities of the pure vitamins together with a "folic acid" concentrate. Through the use of this diet we have attempted to produce specific vitamin deficiencies by withholding the vitamin concerned from the daily supplement. The "folic acid" concentrate (norite eluate according to the method of Hutchings *et al.*[2]) was assayed for its content of each of the B group of vitamins and found to contain between 1 and 2 γ of riboflavin per gram equivalent of original "solubilized liver extract"‡ from which the concentrate was made. The maximum riboflavin furnished by a 5% level of the concentrate was therefore 10 γ. Negligible quantities of riboflavin were contained in the purified casein and in the sucrose used in the diet.

The monkeys were given the basal diet *ad libitum* and all the vitamins except riboflavin were supplied in the daily supplement according to the procedure previously described.[3] Recently obtained monkeys placed on the deficient diet showed growth failure after 6 to 8 weeks while other monkeys

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We are indebted to Merck and Co., Rahway, N.J., for the synthetic vitamins and to Abbott Laboratories, North Chicago, Illinois, for halibut liver oil.

1 Waisman, H. A., and Elvehjem, C. A., *J. Nutrition,* 1943, **26**, 361.

† Smaco vitamin-free casein, obtained from SMA Corp., Chagrin Falls, Ohio.

2 Hutchings, B. L. Bohonos, N., and Peterson, W. H., *J. Biol. Chem.,* 1941, **141**, 521.

‡ Obtained through the courtesy of Wilson Laboratories, Chicago, Illinois.

3 Waisman, H. A., Rasmussen, A. F., Jr., Elvehjem, C. A., and Clark, P. F., *J. Nutrition,* 1943, **26**, 205.

FIG. 1 AND 2.
The "freckled dermatitis" of riboflavin-deficient monkeys.

previously fed various diets containing liver, showed no deficiency until after longer periods of time. In contrast to thiamine-deficient animals, the riboflavin-low monkeys continued to eat and even in advanced deficiency marked anorexia was never observed.

The more significant signs of riboflavin deficiency developed gradually; a "freckled" type of dermatitis appeared first on the face (Fig. 1 and 2) and in the area of the groin; the small, red, dry spots became increasingly prominent and enlarged until they appeared as dark scabs covering nearly the whole area. In more severe cases of the "freckled dermatitis" other areas of the body, notably the arms and legs, became encrusted. The dermatitis occurred at the site of hair growth on the arms and legs, and none was seen at the hairless joints of the fingers and toes. This was in contrast to the presence of dermatitis of the face where a minimum of hair is found.

A well defined incoordination of the limbs was observed in those monkeys which were maintained on the deficient diet for periods as long as 2 to 3 months. The grasping reflex was faulty and the strength both of arms and legs was diminished in 2 of the monkeys.

Some slight variation in the time of onset and in the severity of the symptoms was encountered in the individual animals. In most of the monkeys the first visible sign of the deficiency was the "freckled dermatitis." The deficient animals were pale and apathetic.

Hemoglobin determinations and red and white cell counts showed unmistakable changes from the normal blood picture of monkeys.[3] Table I shows the blood values for 6 of the animals before the development of the deficiency and during the height of the syndrome; the sharp drop in the hemoglobin level was evident shortly after the appearance of the dermatitis. The time at which the lowest values were recorded varied for the different animals from 59 to 108 days after they were placed on the riboflavin-low diet. Another distinctive feature of the deficiency

TABLE I.

Monkey No.	Hemoglobin		Red blood cells		White blood cells	
	Normal value	During deficiency	Normal value	During deficiency	Normal value	During deficiency
	g		millions		thousands	
53	13.4	4.9	5.5	2.0	11.0	8.9
57	14.6	9.7	5.8	2.7	14.0	6.6
64	13.9	6.3	5.28	2.4	13.2	6.42
80	14.9	4.0	4.58	2.3	13.9	7.0
81	13.0	7.72	6.1	3.02	11.0	6.08
82	12.9	8.0	6.09	1.7	13.8	11.8

was the reduced red cell count which usually preceded the decrease in hemoglobin. The white cell count was more variable, but definitely lower than normal values.

Administration of 50 μg riboflavin per day to the deficient animals brought about remission of the early and milder cases of "freckled dermatitis" within 2 to 3 weeks. When the symptoms were of a more advanced nature, however, 50 γ riboflavin per day for 30 or more days were inadequate to give improvement of the animals but higher levels of 100 to 500 γ per day gave remission of all observed signs after 10 to 14 days. The dermatitis cleared rapidly, and "new looking" skin appeared on the hands, feet, and face after several weeks of riboflavin administration.

The time of reappearance of the symptoms after riboflavin was again discontinued was directly related to the level and duration of the administered dosage. When the animals were fed a supplement of 50 γ per day for 3 weeks, they were protected from dermatitis for the following 21 days, while the administration of 500 γ per day to the same animal for 21 days gave protection for approximately 70 days. The deficiency was produced several times in the individual monkeys and each time the animal responded to riboflavin therapy. It has been shown by several groups of workers that the greater portion of the administered vitamins are rapidly excreted and this finding undoubtedly accounts for the disproportionately shorter protective duration with the larger dosage.

Further discussion on the requirement of riboflavin must be deferred until experiments now in progress are completed. Since the diet contained no added biotin it was necessary to ascertain if the dermatitis might be related in part to the secondary biotin deficiency. Accordingly, biotin as a crude concentrate and also in crystalline form were fed both before the onset of the deficiency and during the height of the syndrome with no effect either on the dermatitis or the blood picture. In more recent trials, the riboflavin deficiency signs have been produced in monkeys on diets containing added biotin from the beginning of the experimental period.

It is of interest that no cheilosis or vascularization of the cornea has been observed grossly in these riboflavin-deficient animals. Although 2 of the animals were kept on the low riboflavin diet for 5 and 7 months no readily apparent change in the cornea was observed. The acute type of deficiency produced here is perhaps quite unlike the more chronic and sub-clinical deficiency noted in humans.[4,5,6,7] It should also be recognized that the riboflavin deficiency described in these monkeys is uncomplicated by lack of other vitamins as far as we are able to determine.

Summary. An acute riboflavin deficiency has been produced in monkeys fed a highly purified diet containing all known vitamins except riboflavin. A "freckled dermatitis" and an anemia are the most striking signs of the deficiency syndrome.

[4] Oden, J. W., Oden, L. H., Jr., and Sebrell, W. H., *U. S. Public Health Rep.*, 1939, **54**, 790.

[5] Kruse, H. D., Sydenstricker, V. P., Sebrell, W. H., and Cleckly, H. M., *U. S. Public Health Rep.*, 1940, **55**, 157.

[6] Sydenstricker, V. P., Sebrell, W. H., Cleckley, H. M., and Kruse, H. D., *J. A. M. A.*, 1940, **114**, 2437.

[7] Sebrell, W. H., and Butler, R. E., *U. S. Public Health Rep.*, 1938, **53**, 2282; *Ibid.*, 1939, **54**, 2121.

14463

Inactivity of Coramine (Nikethamide) for *L. arabinosus* and its Conversion to an Active Substance.

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The question of relative activities of various derivatives of nicotinic acid for *L. arabinosus* and for animals has been brought to the front by the discovery of a substance in wheat bran and in certain other materials which is inactive for *L. arabinosus* but is made active by very mild alkali treatment or moderately strong treatment with acid.[1,2] This situation has led to some feeling of unrest in regard to the validity of assay results obtained with *L. arabinosus* using alkali or acid treatment of the samples. Studies to date[3,4] indicate that the animal can utilize derivatives of nicotinic acid more readily than can *L. arabinosus*. The results of the present study support this finding.

Since nikethamide (pyridine β-carboxylic acid diethyl amide) has been found to be quite active for dogs and humans,[5,6,7] we tested it on *L. arabinosus* and obtained 0.03% of the

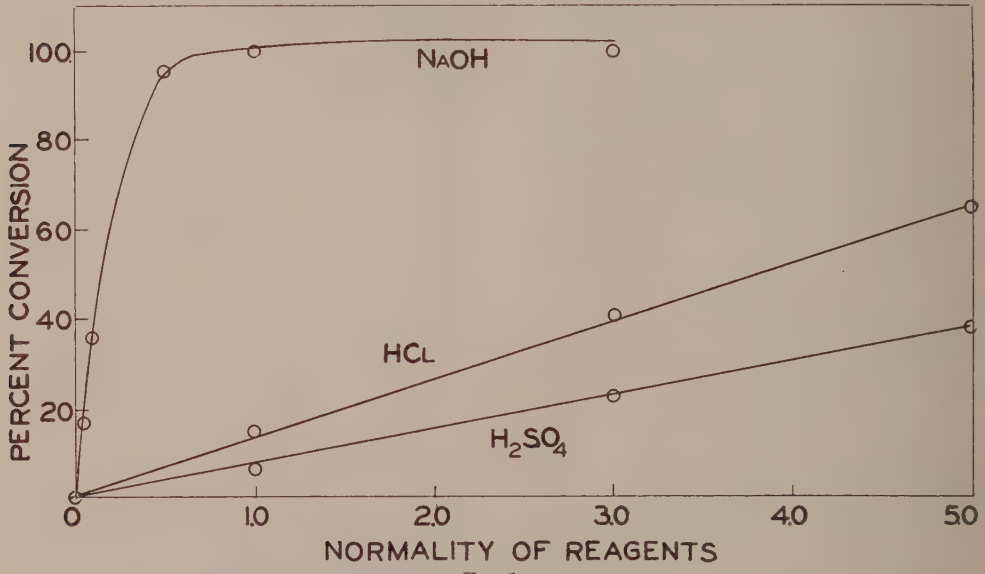

Fig. 1.

Conversion of Coramine to an active substance for *L. arabinosus* by autoclaving 1 hr at 15 lbs with NaOH, HCl, and H_2SO_4.

1 Andrews, J. S., Boyd, H. M., and Gortner, W. A., *Ind. and Eng. Chem., Anal. Ed.*, 1942, **14**, 663.

2 Cheldelin, V. H., and Williams, R. R., *Ind. and Eng. Chem., Anal. Ed.*, 1942, **14**, 671.

3 Elvehjem, C. A., and Teply, L. J., *Chem. Rev.*, in press.

4 Glenn, R. A., Hill, N. C., and Neubeck, V. H., Abs. of papers presented at A. C. S. meeting, Biol. Div., Sept., 1943.

5 Wooley, D. W., Strong, F. M., Madden, R. J., and Elvehjem, C. A., *J. Biol. Chem.*, 1938, **124**, 715.

6 Spies, T. D., Bean, W. B., and Stone, R. E., *J. Am. Med. Assn.*, 1938, **111**, 584.

7 Smith, D. T., Margolis, G., and Margolis, L. H., *J. Pharmacol.*, 1940, **68**, 458.

activity of an equimolar amount of nicotinic acid. This activity may be due to impurities or slight conversion during sterilization. Glenn *et al.*[4] have reported nikethamide to be inactive for this organism. Dorfman *et al.*[8] have found it to be slightly active for *dysentery bacilli*, while Knight[9] and Landy[10] have found it inactive for *Staph. aureus*.

[8] Dorfman, A., Koser, S. A., Reames, H. R., Swingle, K. F., and Saunders, F. J., *J. Inf. Dis.*, 1938, **65**, 163.

[9] Knight, B. C. J. G., *Biochem. J.*, 1938, **32**, 1241.

[10] Landy, M., PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 504.

The compound was autoclaved for one hour with various concentrations of NaOH, HCl, and H_2SO_4 at 15 pounds and the resulting mixtures were assayed with *L. arabinosus*. The results summarized in Fig. 1 show that NaOH is most effective, while HCl is somewhat more potent than H_2SO_4 in converting nikethamide to an active compound. Nikethamide is less easily converted to active material than is the "precursor" found in wheat bran, since autoclaving with 0.1 N NaOH, 1 N HCl, or 1 N H_2SO_4 seems to bring about 100% conversion of the latter substance.

14464

Mechanism of the Diabetogenic Action of Alloxan.*

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Alloxan has been found to produce sustained diabetes mellitus in rats, rabbits, and dogs (Shaw-Dunn and McLetchie,[1] Goldner and Gomori,[2,3] Bailey and Bailey[4]). This diabetes is due to degeneration of the beta cells in the islets of Langerhans (Shaw-Dunn, Sheehan and McLetchie[5]) and consequently to the decrease of insulin production (Goldner and Gomori[6]).

Prior to the establishment of the diabetic syndrome the blood sugar of the rabbit shows a characteristic biphasic reaction, which was first described by Jacobs.[7] An immediate hyperglycemia which reaches its peak within 2 or 3 hours is followed by a severe, often fatal, hypoglycemia, which after a duration of 15 to 20 hours yields to the ultimate hyperglycemia and glycosuria. The hypoglycemic phase is explained by Shaw-Dunn and coworkers[5] as the effect of large amounts of insulin, which are released suddenly from the degenerating beta cells. The phenomenon of the initial hyperglycemia raises the question whether alloxan inhibits insulin before it destroys the site of insulin production. It seemed, therefore, of interest to investigate the question whether insulin is able to prevent the initial rise of the blood sugar after alloxan poisoning and also whether insulin can protect the beta cells against alloxan injury. The latter problem seemed of special interest, since it has been found that insulin treatment and the maintenance of a normal blood sugar level will protect animals against the diabetogenic effect of anterior pituitary

* This work was supported by grants from the Douglas Smith Foundation of the University of Chicago and Eli Lilly and Company, Indianapolis, Indiana.

[1] Shaw-Dunn, J. S., and McLetchie, N. G. B., *Lancet*, 1943, **2**, 384.

[2] Goldner, M. G., and Gomori, G., *Endocrinology*, 1943, **33**, 297.

[3] Gomori, G., and Goldner, M. G., PROC. SOC. EXP. BIOL. AND MED., 1943, **54**, 287.

[4] Bailey, C. C., and Bailey, O. T., *J. A. M. A.*, 1943, **122**, 1165.

[5] Shaw-Dunn, J. S., Sheehan, H. L., and McLetchie, N. G. B., *Lancet*, 1943, **1**, 484.

[6] Goldner and Gomori, to be published in *Endocrinology*.

[7] Jacobs, H. R., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 407.

TABLE I.

Blood Sugar Curves in 10 Rabbits After Intravenous Injection of Alloxan, of Alloxan Plus Insulin, and of Insulin. Rabbits 80, 2, and 16 died in hypoglycemic convulsions after 6, 2 and 5 hours, respectively.

Rabbit No.	Alloxan, mg/kg	Insulin, unit/kg	Application	Blood sugar level: Fasting	1	2	3	4	5	6	24	48
					Hours after injection							
1	200	0	i.v.	108	251	327	—	80	—	28	111	—
62	200	0	i.v.	102	338	378	—	57	—	40	53	460
80	150	0	i.v.	123	305	306	272	—	—	32	—	—
2	200	1	i.v.	82	40	32	—	—	—	—	—	—
16	200	1	into	115	83	61	85	—	37	—	—	—
4	150	1	different veins	107	70	53	75	—	—	55	113	279*
38	200	1	i.v.	111	79	69	69	60	—	—	438	—
18	200	1	mixture	117	65	64	93	84	—	43	—	507
52	150	1		122	139	141	135	—	—	45	—	532
37	0	1	i.v.	121	30	36	39	55	90	115	—	—

*After 72 hours.

extract (Campbell, Haist, Ham, and Best[8]).

Experimental Procedure. Three rabbits out of a series of 6 of the same size and breed were treated simultaneously with a diabetogenic dose of alloxan and with insulin, the 2 substances being injected into 2 different veins, the remaining 3 animals with the same doses of a mixture of alloxan and insulin, kept at room temperature for 60 minutes prior to intravenous injection. A freshly prepared 5% solution of alloxan (Eastman) was used and 150 and 200 mg per kg were given. The insulin dose was 1 unit per kg, a solution of insulin zinc crystals (Eli Lilly and Company) being used. Insulin sensitivity curves and blood sugar curves of rabbits of the same stock injected with the same doses of alloxan alone served as controls. The animals were fasted for 20 hours prior to the experiment. The blood sugar level was determined before the injection and at hourly intervals afterwards. The Miller-Van Slyke[9] micromethod for blood sugar estimation was used.

Results. The results are shown in Table I. The blood sugar curves of rabbits 1, 62, and 80 demonstrate the typical response to a diabetogenic dose of alloxan. The blood sugar level rises immediately after injection, reaches an extreme peak of 300-400 mg % after 2 hours, and falls to a convulsive level soon afterwards. If no glucose injections are given at this time only a few animals will recover to develop hyperglycemia and glycosuria. Rabbit 62 shows such a spontaneous recovery. Rabbit 80 died in hypoglycemic convulsions and rabbit 1 was killed 24 hours after injection. These 3 rabbits are representative of 24 animals which received alloxan injections and followed similar courses. However, the early blood sugar changes were not followed in all of them. At present we have in our series 3 animals with sustained diabetes of more than two months' duration.

The blood sugar curve of rabbit 37 demonstrates normal insulin sensitivity.

The blood sugar curves of the 6 animals which had received injections of alloxan and insulin have in common the fact that they do not show the initial alloxan hyperglycemia. A very slight elevation of the blood sugar is seen in the curve of rabbit 52, negligible if compared with the increase of 200% in the control animals 1, 62, or 80. All other animals show an early hypoglycemic response, which in rabbit 2 was as severe as if insulin alone had been given. This rabbit died in hypoglycemic convulsions. Rabbits 16, 4, 38, and 18 show a moderate fall of the blood sugar within 2 hours after injection. After 3 or 4 hours the blood sugar rises somewhat and seems to be returning to the fasting level. These 4 animals, however, show a second

[8] Campbell, J., Haist, R. E., Ham, A. W., and Best, C. H., *Am. J. Physiol.*, 1940, **129**, 328.

[9] Miller, B. F., and Van Slyke, D. D., *J. Biol. Chem.*, 1936, **114**, 583.

hypoglycemic reaction during the fifth or sixth hour after injection. Rabbit 16 died in this phase in spite of glucose injections. Rabbits 4, 38, 18, and 52 recovered and developed diabetes on the second or third day.

A biopsy from the pancreas of rabbit 52 was taken 5 days after development of diabetes and showed the characteristic changes found after alloxan injection. The beta cells were shrunken or had disappeared completely; the alpha cells appeared normal and seemed to have increased in numbers.

It can be concluded from these observations that alloxan does not inhibit insulin and that the initial alloxan hyperglycemia of the rabbit can be prevented by insulin. Insulin, however, does not protect the islet cells against the injurious effect of alloxan, nor does it prevent the development of alloxan diabetes. The first 4 hours of the blood sugar curves of the 6 rabbits 2, 16, 4, 38, 18, and 52 can be explained as a combination of the hypoglycemic effect of insulin and the hyperglycemic effect of alloxan. In all instances the insulin effect was predominant, since the alloxan hyperglycemia did not appear. In a single instance, however, insulin hypoglycemia was as severe as it would have been if no alloxan had been given simultaneously. The second period of the curves does not differ from the blood sugar curves after alloxan alone. During this period the effect of the injected insulin was diminishing while the hypoglycemic phase of alloxan poisoning had just set in.

Comment. The mechanism of alloxan poisoning is unknown. The experiments here presented give further evidence for the assumption that alloxan affects the beta cells directly and does not act through the medium of disturbed blood sugar regulation. The initial hyperglycemia can be prevented by insulin, the following hypoglycemia by glucose injections, and yet the beta cells will degenerate and diabetes will develop. This is in contrast to the mechanism of pituitary diabetes. Here, the early hyperglycemia, provoked by injections of anterior pituitary extract, is indispensable to the establishment of diabetes, and the maintenance of a normal blood sugar level by whatever means (insulin, phlorhizin, or fasting) prevents the exhaustion of the islet cells and the development of the diabetes. In alloxan poisoning the diabetogenic process will take its course regardless of whether the initial hyperglycemia (which is an accompanying symptom rather than a causal factor) is prevented or not. This has been shown by us also in experiments on dogs, where neither simultaneous and prolonged treatment with insulin nor phlorhizin injections will protect the beta cells against alloxan poisoning.[6] It seems, therefore, that alloxan exerts its effect by direct damage to the beta cells. It does not inhibit insulin, nor does it cause exhaustion of the islet tissue by overstrain.

14465

Experiments on the Toxicity of the Calcium Salt of Penicillin.

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The physical properties of the calcium salt of penicillin make it easier to handle than the deliquescent sodium salt. However, owing to its supposed toxicity,[1] its use is not advised.[2] In the experiments demonstrating its toxicity[1] a crude salt containing 50 units/mg was used. Ten mg given intravenously and 20 mg given subcutaneously caused severe but not fatal reactions in 20 g mice. In previous experiments[3] it had been shown that the crude sodium salt containing 40 to 50 units/mg caused "serious embarrassment" when injected into mice. With purification the sodium salt becomes relatively less toxic;[1] 20 mg of the salt containing 250 or 325 units/mg produced no effect in 20 g mice. There is reason to believe that the calcium salt might undergo a similar detoxification and these experiments were done to verify this.

In the early experiments of the Oxford group[1] the calcium content of the preparation has not been determined. The fact, however, that even 10 mg of crude salt given to mice intravenously proved to be toxic, makes a direct correlation with the calcium content of the salt very improbable. It is a safe assumption that the calcium content of such a crude preparation must have been under 10%, or, in other words, less than 1 mg in 10 mg of the salt used.

Method. Two samples of freeze-dried calcium salt were used,† A contained 400 units/mg and B 230 units/mg. The calcium content of salt B was 1 mg per 5,000 units. The salts were dissolved in sterile distilled water and injected into mice intramuscularly and intraperitoneally. The intramuscular injections were made into the left scapula region. Each salt was dissolved in sufficient water that the required dose was contained in 0.01 cc per gram of mouse.

The mice used weighed between 13 and 28 g. They were observed frequently within the first 3 hours of injection and again approximately 12 and 24 hours after injection. Those mice which were injected intraperitoneally in the first experiment were injected intramuscularly in the second and vice versa.

Results. Experiment 1. Salt A was used. Dose: 50 units per gram of mouse. Ten mice were injected intraperitoneally and 10 were injected intramuscularly. The mice showed no reaction within 24 hours of injection

Experiment 2. Salt B was used. Dose: 100 units and 0.02 mg calcium per gram of mouse. Ten mice were injected intraperitoneally and 10 intramuscularly. The mice showed no reaction within 24 hours of injection.

The observations were continued for several more days, but no signs of local irritation or other toxic signs were noticed. More concentrated solutions of the salts could not be produced and therefore higher doses could not be given. The dose employed in the second experiment is approximately 50 times that at present used in human therapeutics as the daily dose of the sodium salt on a weight for weight basis.

No toxic effects were noted in the mice after intramuscular injection in spite of the excessive doses used.

The salts A and B and another sample containing 280 units/mg were used in humans without ill effect. Aqueous solutions of the

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[1] Florey, H. W., and Jennings, M. A., *Brit. J. Exp. Path.*, 1942, **23**, 120.

[2] Florey, H. W., and Florey, M. E., *Lancet*, 1943, **1**, 387.

[3] Abraham, E. P., Chain, E., Fletcher, C. M., Gardner, A. D., Heatley, N. G., Jennings, M. A., and Florey, H. W., *Lancet*, 1941, **2**, 177.

† Preparations obtained from Reichel Laboratories, Kimberton, Penn.

salts were given to 9 patients in intravenous, intramuscular, and even subcutaneous application. In 2 cases 80,000-100,000 Oxford units were administered in constant intravenous drip for 2 x 24 hours. The doses given intramuscularly (6 to 8 times within 24 hours) ranged from 5,000-20,000 units (1-3 cc) and were equally well tolerated without local or systemic reaction.

Conclusion. Satisfactorily purified Ca-salt of penicillin is not more toxic for man and mice than its Na-salt.

14466

Impairment of Growth and Myelinization in Regenerating Nerve Fibers Subject to Constriction.*

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A nerve scar is known to impede the passage of regenerating nerve fibers. However, whether or not fibers that succeed in getting through a constriction remain handicapped in their further development has never been determined. The experiments to be described in the following prove that they do.

Material and Method. In 11 white rats (120-230 g) the sciatic nerve was crushed *cca.* 5 mm above its bifurcation. The tibial and peroneal nerves were cut at the knee, and a segment of artery, small enough to produce local nerve constriction, was slipped over one of the proximal stumps, as described previously.[1,2] Fig. 1 illustrates the operation (C, crush; S, constriction sleeve). Nerve regeneration is thus started simultaneously in both nerves at level C. Fibers of the constricted nerve must grow through the bottleneck, while fibers growing through the unconstricted stump serve as controls. After regeneration periods of from 4 to 15 weeks, the nerves were fixed and stained in osmic acid or in Protargol (Bodian) and Mallory Azan. Fiber diameters were measured and compared at levels both proximal (P) and distal (D) to the constriction sleeve.

Results. In 3 cases the sleeves had failed to constrict. No difference between test and control nerves was noticed; hence, mere presence of a sleeve does not affect regeneration. In the remaining 8 cases, constrictions of varying degrees had persisted, associated as usual with edema and with damming of axoplasm.[2,3] Numerically, fibers have regenerated as extensively through the constricted as through the unconstricted nerves. They contrasted sharply, however, with regard to caliber and myelinization. The fibers that had passed through the constriction were markedly thinner and delayed in myelinization as compared with the regenerated fibers of the control nerve at the same level.

Photographs of representative samples from cross-section D of nerve pairs regenerated with (A) and without (B) constriction, taken at identical magnifications 8 weeks (Fig. 2) and 10 weeks (Fig. 3) after the operation, illustrate the differences. A lower power view (Fig. 4) of both the test (tibial; bottom) and control (peroneal; top) nerves shows the contrast in myelinization (osmic acid stain).

In one case (R 188; 6 weeks regeneration time), all regenerated fibers of the constricted tibial and the unconstricted peroneal (total: 5997 fibers) were measured. Their size distribution is given in Table I. While 50%

* The work described in this paper was done under a contract, recommended by the Committee on Medical Research of the Office of Scientific Research and Development and the University of Chicago, and was also aided by the Dr. Wallace C. and Clara A. Abbott Memorial Fund of the University of Chicago.

1 Weiss, P., *Arch. Surg.*, 1943, **46**, 525.

2 Weiss, P., and Davis, H., *J. Neurophysiol.*, 1943, **6**, 269.

3 Weiss, P., *Anat. Rec.*, 1943, **86**, 491.

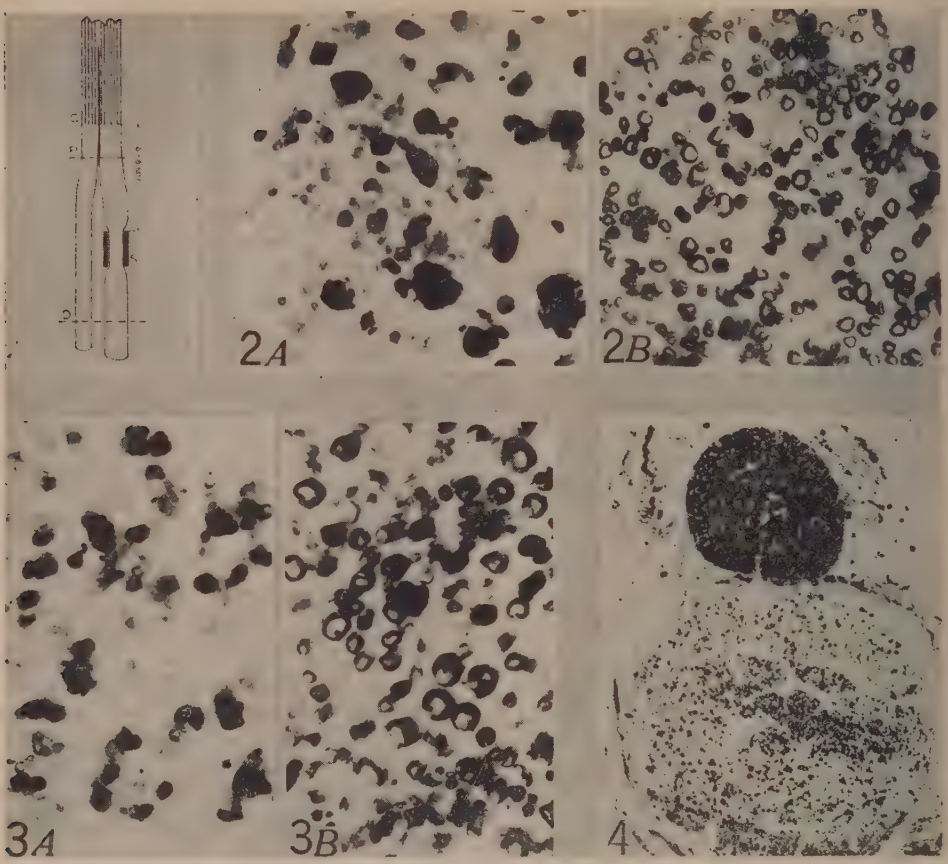

FIG. 1. (Top left) Diagram of operation.

FIG. 2. Cross-sections at level D through the regenerated branches of a sciatic nerve crushed at C, the tibial branch (A) having regenerated through a constriction S, the unconstricted peroneal (B) serving as control. Case R 191; regeneration time 8 weeks. Osmic acid. 450 ×.

FIG. 3. Same as Fig. 2 of a case (R 138) of 10 weeks' regeneration time. Osmic acid. 450 ×.

FIG. 4. Total view of the nerves of Fig. 3 (R 138) at level D, showing the difference in myelinization between the constricted (bottom) and unconstricted (top) nerves. Osmic acid. × 48.

TABLE I.

Size Distribution of the Total Population of Regenerated Fibers in the Constricted and Control Nerves of R188 (6 weeks p.op.), Measured at Level D (Fig. 1) Distal to Constriction.

Nerve	Total No. of fibers	% distribution by equidistant size classes*					%	
		I	II	III	IV	V	<3 μ	>3 μ
Control (Peron.)	2350	2.0	47.8	37.9	10.2	2.1	49.8	50.2
Constr. (Tibial)	3647	10.2	80.3	9.5			90.5	9.5

*Class I contains fibers below 1.5 μ, class V those over 6.7 μ, with classes II-IV intermediate at equal intervals.

TABLE II.
Size Distribution and Average Diameter of the 150 Largest Regenerated Fibers in Each Nerve Proximal and Distal to Level of Constriction in R188 (6 weeks p.op.) and R138 (10 weeks p.op.).

Case	Nerve	Level (Fig 1)	Size classes* 4	5	6	7	8	9	10	11	12	13	14	15	16	Diameter Range μ	Diameter Avg μ
	Control	P				69	58	23								5.2- 7.8	6.7
	(Peron.)	D			14	88	38	10								4.3- 7.8	6.3
R188 (6 wk)																	
	Constr.	P						66	58	22	4					6.5-10.4	*8.5*
	(Tib.)	D	50	85	15											2.6- 5.2	*4.1*
	Control	P						86	48	12	4					7.0-10.4	8.3
	(Peron.)	D					57	46	37	10						6.1- 9.6	7.8
R138 (10 wk)																	
	Constr.	P									25	67	43	12	3	9.6-13.9	*11.6*
	(Tib.)	D		113	37											3.5- 5.2	*4.5*

* Class 4 ranges from 2.6 to 3.5 μ, subsequent classes following in steps of 0.87 μ.

of the control fibers had a diameter of more than 3 μ, fewer than 10% of the constricted fibers had attained that size. In this and another case (R 138; 10 weeks) the 150 largest fibers of both the constricted (tibial) and the control (peroneal) nerve were measured and are recorded in Table II.

The table reveals: (1) Proximal to the constriction (level P), the regenerated tibial contains larger fibers than the regenerated peroneal. (2) Distal to the constriction (level D), the tibial fibers are greatly reduced in size, both relative to the peroneal fibers of the same level, and even more so, relative to their own proximal parts. (3) A consistent shift in the fiber spectrum of the control nerves between levels P and D proves that regenerated fibers taper proximo-distally. (4) Fibers in the control nerve and the unconstricted portion of the test nerve are larger in R 138 than in R 188, reflecting the additional 4 weeks regeneration period of the former. Significantly, the test fibers beyond the constriction do not show a corresponding gain. This indicates that constricted fibers are permanently retarded, a view supported by 2 cases studied after 15 weeks, in which the differential between the 2 nerves was found undiminished. Terminal results after still longer regeneration periods will be reported on a later occasion.

Discussion. Discussions of nerve regeneration have in the past centered on the outgrowth phase of the regenerating axon. The further elaboration of the fiber, fittingly referred to as "maturation,"[4] has received less attention. Our present experiments bear on this latter phase. They prove that a localized constriction deprives that part of a regenerating fiber lying beyond it of some factor essential for its further growth in width and myelinization. Evidently, a growing fiber requires continuous contributions from its central cell body, the throttling of which entails a corresponding reduction of growth and myelinization. The damming of axoplasm in front of constrictions, to be detailed in a forthcoming article, suggests a translatory movement of axoplasm as the mechanism involved. This process differs from the substance convection within axons advocated by Gerard[5] and Parker,[6] without excluding it. It would explain the "vis a tergo" of Held and the "formative turgor" of Cajal.

According to our results, the size of any cross-section of a fiber will have a bearing on the size of the same fiber at farther distal levels. Consequently, a small nerve fiber regenerating into a large stump cannot be expected to fill the latter to full size.[7] The condensation of scar tissue in and around suture lines will act much as did our experimental constrictions. Whether the rate of

4 Young, J. Z., *Physiol. Rev.*, 1942, **22**, 318.

5 Gerard, R. W., *Physiol. Rev.*, 1932, **12**, 469.

6 Parker, G. H., and Paine, V. L., *Am. J. Anat.*, 1934, **54**, 1.

7 Nageotte, J., and Guyon, L., *C. R. Soc. Biol.*, 1918, **81**, 571.

lengthwise extension of growing fibers is also affected by constriction, remains to be determined; if it is, the puzzling fact that the rate of advance of nerve fibers inside the distal stump is faster after crushing (precluding scars) than after suture (with some scar formation),[8] would find a ready explanation.

In practical regards, the demonstration of the additional hazard of scar contraction only underlines the value of measures designed to eliminate scar formation. In saying this, we take the desirability of complete caliber recovery and myelinization for granted, although their relevance for functional recovery has never been accurately determined.

Summary. Nerve fibers regenerating in a nerve with localized constriction are greatly reduced in caliber and delayed in myelinization at all levels distal to the constriction. Scar tissue, therefore, presents not only a local impediment to the outgrowth of regenerating fibers, but a continued hazard to their maturation.

[8] Gutmann, E. J., *Neurol. and Psych.*, 1942, **5**, 81.

14467

Acute Toxicity of Mercurial Diuretics.

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The present experiments were performed to compare the acute toxicity of the mercurial diuretics, and to study the effect of theophylline and the speed of intravenous injection upon the acute toxicity.

Cats were used as test animals. Eight materials were studied: (1) mercuric chloride, (2) mercuric chloride mixed with theophylline, (3) mercurin, (4) mercupurin (of commerce), (5) salyrgan, (6) salyrgan-theophylline (of commerce), (7) salyrgan freshly mixed with theophylline, (8) same as (7) but allowed to age for a month at room temperature. The acute toxicity was determined by the amount of mercury necessary to cause death in unanesthetized cats by frequent, regularly spaced intravenous injections.

Results. The results are summarized in Table I. They show that the acute toxicity of mercury is essentially the same whether administered in the form of mercuric chloride, mercuric chloride with theophylline, or salyrgan; namely, the fatal dose is approximately 15 mg Hg per kg.

However, mercury is only about one-half as toxic when administered in the form of salyrgan-theophylline, mercupurin, and mercurin; *i.e.*, the fatal dose is about 30 mg Hg per kg. These results fail to confirm those of DeGraff and Lehman[1] who found that the toxicity increased in the order mentioned.

It is clear that theophylline does not alter the toxicity of all mercurials since the mercupurin which contains the theophylline has the same toxicity as the mercurin which is without theophylline, and the mercury in mercuric chloride has the same toxicity with or without theophylline.

In the case of salyrgan, however, theophylline does alter the toxicity. It reduces the toxicity to one-half. Such an influence of theophylline was recently reported by DeGraff and Lehman[1] who ascribed the change to a chemical reaction which occurred when the 2 drugs were mixed. Our results also favor some form of reaction between them because time is required before the mixture will exhibit lower toxicity than the salyrgan alone. When the test was made directly after the 2 compounds, salyrgan and theophylline were mixed in our laboratory, the toxicity of the mercury was the same as that of salyrgan alone. However, when the mixture was allowed to stand for a period at room temperature, its toxicity was reduced to

[1] DeGraff, A. C., and Lehman, R. A., *J. A. M. A.*, 1942, **119**, 998.

TABLE I.
Lethal Dose of Mercurial Diuretics in Cats.

Rate of injection	$HgCl_2$ 0.1%	$HgCl_2$ 0.1% and Theophylline 0.05%†	Mercurin	Mercupurin	Salyrgan	Salyrgan-Theophylline (commercial preparation)	Salyrgan mixed with Theophylline same proportions as commercial preparation: freshly mixed	Salyrgan mixed with Theophylline same proportions as commercial preparation: one month old
0.4 mg Hg/kg/min.	24.8	12.0	12.8	12.8	11.2	30.1		
	15.9	11.6	22.5	13.6	22.3	37.8		
	21.8	31.3	24.8	37.6	7.9	25.6		
	15.9	22.6	40.2	34.3	12.6	33.9		
	7.7	12.7	55.3	45.0	28.1	31.2		
Avg	17.2	18.0	31.1	28.7	16.4	31.7		
S.E.	±2.9	±3.9	±6.1	±6.5	±3.8	±2.0		
0.8 mg Hg/kg/min.	9.6		31.0	40.0	21.0	37.4		19.2
	12.8		31.4	33.9	14.4	44.3		28.8
	12.8		24.2	11.3	12.6	28.5		44.8
	19.2		7.9	11.3	11.4	40.5		35.2
	30.4		33.4	35.0	8.3	30.6		19.2
Avg	17.0		25.6	26.3	13.5	36.3	14.8*	29.4
S.E.	±3.7		±4.7	±6.2	±2.1	±3.0	±1.6	±4.9
1.6 mg Hg/kg/min.	20.4	10.2	34.9	12.8	9.9	7.9		
	10.2	13.6	45.1	35.4	13.0	17.7		
	22.3	10.2	41.3	35.2	9.4	32.0		
	6.8	21.8	12.8	41.3	8.0	22.1		
	6.8	13.6	28.8	38.4	29.1	36.2		
Avg	13.3	13.9	32.6	32.6	13.9	23.2		
S.E.	±3.4	±2.1	±5.7	±5.1	±3.9	±5.1		
Grand Avg	15.8	16.0	29.8	29.2	14.6	30.3	14.8	29.4
S.E.	±1.9	±2.2	±3.3	±3.3	±1.8	±2.4	±1.6	±4.9

Each figure represents one cat. All values are expressed in terms of mg Hg per kg. Concentration of elemental mercury in all the organic preparations as diluted for injection is the same: 1.6 mg Hg per cc.

* This average figure was obtained on 10 cats, injected at the rate of 0.8 mg Hg per kg per min., dying of 20.8; 21.1; 16.0; 9.6; 11.4; 8.0; 9.6; 14.4; 20.8; and 16.0 mg Hg per kg, respectively.

† Theophylline ethylenediamine was used.

that of the preparation of commerce, salyrgan-theophylline. No visible changes in the solution occur on standing. We have no data on the rate at which the change takes place. In view of the influence of aging, it will be necessary to test other mercurials in that way before the matter of the effect of theophylline on toxicity is decided.

In relation to the effect of the speed of injection on toxicity, DeGraff and Lehman[1] observed that the lethal dose was smaller the slower the rate of injection. Such a phenomenon is often seen in the case of drugs which develop their actions slowly so that in the course of rapid injection one may overrun and secure a value which is larger than the true minimal lethal dose. That being the case, one may question the inference which the authors made that the drug is less toxic when given rapidly. It may also be noted that our results fail to show any significant or consistent difference in the lethal dose within wide variations in the rate of administration of the mercurials examined. In the study by DeGraff and Lehman,[1] the rates of injection varied from 1 to 10 mg Hg per kg per minute, so that death was produced in from 3 to 7.5 minutes in the group receiving the mercupurin

most rapidly. In our study, the mercurial was injected far more slowly, from 0.4 to 1.6 mg Hg per kg per minute. Values approximating ours for the lethal dose of mercupurin, mercurin, salyrgan, and salyrgan-theophylline were obtained by DeGraff and Lehman,[1] when they injected the mercurial slowly (1 mg Hg per kg per minute). This favors the conclusion that in their titrations the actual acute lethal dose was overrun, in the case of their rapid injections, in proportion to the speed of administration.

Conclusions. (1) The admixture of theophylline with salyrgan reduces the toxicity of the latter as the result of some reaction between the two compounds inasmuch as aging is required before the changed toxicity is demonstrable. (2) This influence of theophylline did not apply to mercurin. (3) In terms of mercury, non-ionizable organic mercurials are not necessarily less toxic than mercuric chloride.

14468 P

Cultivation of *Trypanosoma gambiense in vitro* in Cell-Free Medium.*

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The diagnosis of African Sleeping Sickness has depended upon confirming clinical impressions by a direct demonstration of trypanosomes in the blood, lymph node fluid, or spinal fluid of the patient or else indirectly by animal inoculation. It seems that many infected cases might be overlooked by these methods, a particularly unfortunate circumstance in the early stages of the disease when treatment is usually so very effective.

In this preliminary paper it will be shown that it is easy to cultivate *Trypanosoma gambiense,* and that by this means infection may be demonstrated when other methods fail. Amongst previous investigators of this problem there is space here to mention only Brutsaert and Henrard[1] whose brilliant and successful work with cultures at Leopoldville, Belgian Congo, was a marked stimulus.

Two strains of *Trypanosoma gambiense,* both originating in Liberia, were used.† Both behave similarly in cultures.

The culture medium has the following formula: Part a: Sodium chloride 8 g, nutrient agar 1.5% (Difco) 4 g, distilled water, to make 900 cc; Part b: Human plasma (citrated) 100 cc, human hemoglobin 20 cc.‡

The base (part a) is sterilized in the autoclave, and part b added later. The medium is dispensed in test tubes and corked with rubber stoppers. The final pH is 7.4-7.5. It will be seen that the medium is a modification of that used by Noguchi and Battistini[2] for the cultivation of *Bartonella bacilliformis.*

Cultures are incubated at room temperature (26-28°C), and become positive 7 to 10 days after inoculation, even when the original inoculum contains no microscopically demonstrable trypanosomes, but the tubes should not be discarded until after one month. The cultures remain viable for 60 days or more, the maximum thus far observed being 71 days. After 2 weeks of cultivation, the pH of the medium drops to 7.2.

Thus far isolations from inoculated susceptible animals have been regularly successful.

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† Obtained through the kindness of Dr. Henry D. Murray and Dr. G. G. Campbell.

‡ Made by laking 1 part of blood with 3 parts of distilled water.

1 Brutsaert, P., and Henrard, C., *C. R. Soc. de Biol.,* 1938, **127**, 1469.

2 Noguchi, H., and Battistini, T., *J. Exp. Med.,* 1926, **43**, 851.

This result was obtained when the donors were in a "negative period" and indeed even when no trypanosomes had ever been microscopically demonstrable. Thus *M. mulatta* 43104, inoculated September 3, became infected. On September 29 and October 4 trypanosomes were seen, and on each of those days blood of this monkey was inoculated by the intraperitoneal route into rat 43111F. The trypanosomes were never found in the blood of this animal in 29 daily examinations from October 4 to November 2. However, cultures made on October 18 became positive and proved that the rat had become infected.

That the cultural method is more sensitive than the animal inoculation test for detecting light infections is also indicated by the following: Monkey 43104 did not react positively following the inoculation of a killed trypanosomal antigen on October 12 at which time *T. gambiense* was no longer demonstrable in the blood. The question arose whether this result could be attributed to eradication of the infection. On October 21 the animal was bled and two 140-g rats (43119) received respectively 1.0 cc and 0.5 cc of the blood intraperitoneally. During one month and a half no trypanosomes were found in the blood of either animal. Conversely, cultures inoculated with 0.3 cc of the same blood were positive in 10 days.

Cultures have now been maintained through numerous transfers for a period of 127 days. Following each transfer multiplication takes place with the production of numerous dividing forms so that unlimited survival *in vitro* seems not unlikely.

Multiplication takes place by the longitudinal division of trypanosome forms into 2 or more daughter individuals. Rosettes or clumps of trypanosomes are frequent. Quite common are very slender organisms with a narrow undulating membrane measuring, after staining, only 1.5-2.0 μ in greatest width; these appear to correspond to the proventricular forms seen in the vector (*Glossina*). Another form is easily twice this diameter, possesses a wide conspicuous undulating membrane, and there are various intermediaries between these two. Multiple division forms composed of from 3 to 6 or more individuals also resemble forms described in Glossina. Another peculiar type are trypanosomes with an enlarged globular posterior extremity.

Summary. 1. *Trypanosoma gambiense* is readily cultivated on a semi-solid medium containing human plasma and hemoglobin. 2. Infection can be detected by cultures, when the usual methods of examination and inoculation of the blood fail. 3. Successful numerous transfers, each followed by multiplication, suggest that the cultures may be able to be maintained indefinitely.

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